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HIGHLY SENSITIVE FIBER-BASED DEVICES FOR GENE AND PROTEIN ANALYSIS

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HIGHLY SENSITIVE FIBER-BASED DEVICES FOR GENE AND PROTEIN ANALYSIS

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ABSTRACT

Single cell probing has found a number of applications in different areas of research. It can help us to better understand cell-to-cell interactions; it has found numerous applications in immunology, cancer research, detection of pathogenic infections and genetic abnormalities. The single cell analysis is very important in stem cells research and development of cells. The main obstacle in the single cell analysis is the small amount of analyte that a single cell could provide. Another difficulty is connected to the cell-to-cell variability inside the uniform population due to the differences of single cells in size, activity, mitotic stage, and functions.

To overcome these problems several methods of single cell analysis including utilization of fluorescence and microfluidic platforms were developed. However, existing methods for cell isolation and analysis are laborious, costly, taking long time and often lack sensitivity, which is necessary for a single cell level probing. Therefore there is a need for novel high throughput and convenient experimental techniques for analysis of gene expression in individual cells. Fiber-based devices with high surface area that are modified with mRNA capturing element could be an attractive candidate for this purpose. We proposed to develop microfluidic electrospun fiber yarns based biosensors for applications in detection of small amounts of targeted analytes, such as mRNA material of a single cell or specific secondary antibodies molecules.

In order to achieve this goal of improving single cell analysis we produced highly sensitive microfiber probes for PCR analysis of mRNA present in concentrations

corresponding to those in a single cell. The bundle of microfibers with high specific surface area was modified with oligo dT_{25} which specifically binds the poly-A tail of mRNA. Now that RNA is captured by the fiber, relative expression of β-actin by contractile and synthetic vascular smooth muscle cells, as well as by adult and neonatal myocytes, was used as a model to evaluate performance of the fiber-based devices for mRNA extraction. Application of fiber yarns as the mRNA isolation probes for PCR analysis improves usability during the sample collection and processing and allows for focused probing from a desired area.

The sensitivity of an enzyme-linked immunosorbent assay (ELISA) performed directly on the fiber-based devices was also evaluated. Electrospun fiber yarns were covalently coated with a model capture antibody and then used to evaluate presence of the specific secondary antibodies in solutions. The colorimetric ELISA procedure was performed with ABTS substrate to investigate sensitivity of prepared sensors in the ELISA application.

Overall, these fibers provide a new flexible platform for a number of analysis types that can considerably improve its convenience and availability for various applications. This approach is universal and could be utilized to develop fiber-based devices that could be potentially used in point-of-care devices and for forensic analysis.

DEDICATION

I would like to dedicate this work to my family: my wife Raisa and my parents Irina (mother) and Dorzha (father), who supported me throughout my life and helped me to reach my goals.

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CHAPTER 1

INTRODUCTION

My graduate research at the Department of Bioengineering in Clemson University focused on the development and implementation of fiber-based biosensors for various applications. The goal of this study was to design a highly sensitive fiber-based probing device for mRNA extraction from various tissue samples and lysates, including individual single cells. Chapter 2 of this dissertation presents a detailed review of the existing literature on the single cell isolation and analysis methods, their advantages and drawbacks and also discusses single dimensional based biosensors and their applications.

Chapter 3 presents overview of the background and clinical significance and lists specific aims that were proposed in current study. Model studies for the use of fiber-based devices in the application for mRNA extraction or development of ELISA-based testing system are discussed in this chapter.

We performed preparation of fiber yarns by electrospinning method from several compositions of polymers mixtures following with characterization of prepared fiber bundles. Physical properties of fiber yarns such as permeability, porosity and surface area were estimated. Fiber yarn modification procedure was established and included several steps of consecutive treatments with different reagents and washing buffers. Total biomolecules loading was investigated by measuring covalent attachment of streptavidin molecules to the surface of fiber yarns following with attachment of oligo dT_{25}

complexes. Fiber yarns biosensors preparation and modification procedures are described in the Chapter 4.

Testing of the prepared fiber-based devices with the samples contained mRNA (tissue, cell lysates, and individual single cells) was performed and compared to the conventional methods of analysis – using commercially available columns and oligo dT coated magnetic beads for mRNA extraction. Chapter 5 covers procedures for mRNA extraction from various samples, comparison of analysis methods, explanation for the choice of materials, and determination of the lowest sensitivity limit for the fiber-based devices. A pilot experiment that modeled extraction of mRNA from tissues *ex vivo* was carried out and discussed in this chapter. Several experiments for analysis of individual single cells separated by flow cytometer are discussed in the chapter 6. Samples that contained single cells as well as those contained tens of cells were analyzed.

The approach of fiber yarns modification is universal and could be also utilized in other potential methods of microbiological analysis. Chapter 7 is focused on the design of fiber-based devices that could utilize one of the most sensitive methods of detection of different pathogens and antibodies - ELISA. We compared specific capture antibodies coated fiber yarns performance in detection of secondary antibody in solutions to the control antibody coated yarns and conventional assay format. Thus, polymeric fiber-based devices prepared by the electrospinning method are capable of performing detection of various analytes – from RNA molecules to proteins.

CHAPTER 2

LITERATURE REVIEW

Single cell probing has found applications in several fields of study, such as neuroscience, [1] development of cells, [2] immunology [3] and cancer research. [4] For example, in cancer research this method could be useful to find a single cancer cell in the tissues detecting cancer at the early stage. Availability of more cell-type-specific information is expected to lead to a much better understanding of physiology in such diverse areas as embryonic development, aging, cell-to-cell communication, pathogen defense, or abiotic stress response. [5] It could also be critical in understanding of pathophysiology of a number of genetic conditions.

Performing single cell probing, one faces many obstacles, one of the biggest problems being the small amount of analyzed material. The easiest target for detection in the single cell is the genetic material, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), which could be amplified by various methods. Genes' expression levels could provide information about individual cell viability, metabolism and structural changes of the cell and could be indirectly used as an indicator for proteins expression levels. Analysis of single-cell gene expressions can lead to a better understanding of human disease pathogenesis and important diagnostic applications. [6]

For analysis of single cell gene expression levels, researchers have to isolate the cell first, which is a complicated process. Separation of desired cell type is achieved by

using one of the following methods: flow cytometry and its specialized type - fluorescence activated cell sorting (FACS), [7] micro pipetting using glass capillaries, laser techniques such as optical tweezers and laser capture microdissection (LCM), [8] and techniques using microfluidic devices. Flow cytometry and microfluidic devices require introducing of a fluorescent label on cells and special instrumentation, while micro pipetting and laser techniques require use of micromanipulator and a microscope. Overall, all of these methods are laborious and require a trained person to operate instruments.

The genetic material from an isolated single cell can be analyzed using one of the following techniques. The DNA or RNA detection usually involves use of fluorescent based methods, such as Förster Resonance Energy Transfer (FRET), Fluorescence *in Situ* Hybridization (FISH) or using fluorescent nanoparticles and quantum dots. These methods use the genetic material from a single cell without amplification, and therefore their sensitivity is limited. The most sensitive method for DNA or RNA detection is the amplification technique called polymerase chain reaction (PCR) and its modifications, such as real time quantitative PCR (RT-qPCR) and single step reverse transcription PCR (RT-PCR). However, single cell PCR also has a number of limitations. The automated single cell PCR method is costly and requires special equipment and materials, as well as a specially trained operator, while the conventional multi-tube PCR analysis shows low sensitivity because of contamination or low retention of genetic material. [9]

Recently, single cell analysis was performed by using different kinds of probes. There are several publications on the single cell probing using the glass pipette with attached carbon nanotube, [10] or the endoscope based on the optic fibers with attached SnO_2 nanowire. [11] Both of these methods utilize a one-dimensional (1D) probe for piercing the cell and collecting the genetic material. Use of 1D structures is favorable for such sensors because these structures possess high surface area, can be readily modified and easily manipulated. However, these methods are still under the early stages of development and require more investigation.

Thus, there is an apparent need for novel high throughput and convenient experimental techniques for analysis of gene expression in individual cells. Our research is focused on the preparation of modified fiber-based devices for mRNA extraction from single cells. We expect that use of such devices will enable researchers to perform quick analysis of individual cells in complex systems, such as tissues comprising of several different cell types. Such fiber sensor could possibly find an application in non-invasive biopsy, or in detection and recognition of a number of types of pathogenic bacteria.

The goal of this review is to evaluate existing literature about single cell isolation and analysis techniques. It covers description for each method of isolation and analysis and highlights their advantages and drawbacks. Applicability of fiber-based devices for the single cell probing is also discussed.

2.1. SINGLE CELL ISOLATION METHODS

Currently, there are several single cell isolation methods employed: flow cytometry, using micromanipulators with a glass capillary, using laser optical tweezers, microfluidics and microarray devices. Each of these methods has their own advantages and disadvantages that are discussed below.

2.1.1. Flow cytometry

Flow cytometry is the most commonly used method of separation of single cells using the fluorescent label on the surface or inside the volume of cells. Flow cytometry allows separation of thousand single cells per minute based on their size, granularity and fluorescent properties. This method is used for many different applications, several of them are: detection of viability and apoptosis, total DNA/RNA content, detection of different antigens, different proteins expression and localization. [12] Recent advances in the instrumentation base allowed creating cell sorting devices, based on fluorescence activated cell sorting (FACS). This method allows separating single cells based upon the specific light scattering and fluorescent characteristics of each cell. The method is capable of sorting thousands cells per minute with capability of isolation of small cell populations with desired internal structure.

There are several flow cytometry instruments available on the market from BD Biosciences, DAKO and Life Technologies that are capable for isolation of single cells.

The single cells isolation could be performed and single cells deposited into the wells of 96 or 384 PCR well plates, which allows analyzing many cells at the same time.

A recent paper on isolation of algae cells for biodiesel production used FACS with lipid soluble Nile Red dye. [13] By incubation of algae population in the greenhouse light and then sorting population of cells that possess highest lipid production several times during 38 days, the authors reached 5-fold lipid production increase by algae cells. These cells could be used to produce biodiesel of high purity and in shorter times.

In the paper by Singhatanadgit W. et al. [14] the authors were using flow cytometry to isolate several different clones from periodontal ligament tissue. These periodontal stem cells were different by proliferation rate, and by the differentiation routes. The authors found that clone C7 was the most potent cell line and it could be used for bone formation *in vitro*, suggesting that such cells may have potential value for stem cell-based bone tissue engineering *in vivo*.

FACS is being extensively used for isolation of bacteria population from soil samples. [15] The authors used *in vitro* and *in vivo* compartmentalization to maintain a linkage between a protein, the gene that encodes it and the activity of that protein. *In vitro* compartmentalization (IVC) is a technique that has been developed to maintain this association by creating cell-like structures where single genes are encapsulated in an artificial membrane along with the components required to transcribe and translate them, plus any additional substances such as enzyme substrates. [16] The FACS method allowed different group to isolate 95% of compartments that contain a single cell. [17]

Flow cytometry, especially FACS method is a very powerful and versatile tool for single cell isolation. This method allows simultaneous isolation of huge number of single cells in a short period of time. Sorting of cells could be done depending on their fluorescence, size or complexity of internal structure. However, there are several drawbacks of this single cell isolation method. First, flow cytometers require careful calibration with significant amount of cells, and most of cells are wasted by the instruments. Second, there is a need to carefully choose the components of cell suspension mixture, as they can interact with downstream reaction if using the PCR for amplification of signal. Third, these instruments require significant investment and are costly to maintain. Moreover it is not possible to perform the dynamic analysis of single cells and observe spatial localization of fluorescence within a cell.

2.1.2. Glass capillary

This method of single cell isolation requires an inverted phase contrast microscope, mounted with micromanipulators. [18] The isolation of cells is performed from the bottom surface of petri dishes with adherent cells by using glass capillaries. The diameter of the capillary tip supposed to be at ~10 μm level, to ensure that it is capable of taking whole cell without damaging it. Pipettes should be emptied in standard PCR tubes containing 2 μl lysis solution. This small volume allows high concentration of detergents needed for cell lysis and RNase inhibition. Careful breaking of the glass tip against the bottom of the tube facilitates complete emptying of the capillary.

The alternative method is to use patch-clamp capillary (diameter of the tip ~0.5 μm) to collect only cytoplasm of the cell. [19, 20] After isolation of cell or cytoplasm, the material usually deposited in the 0.2 ml PCR tube for future analysis. The advantages of this method are collecting the single cells from intact tissue and capability to select cells by phenotype and viability. However, this method is very challenging, which limits the number of cells that can be collected in short period of time.

2.1.3. Laser capture

A recently developed laser capture microdissection (LCM) technique [21, 22] allowed researchers to isolate cells of interest from the tissue section using a laser that is coupled with a microscope. The tissue section could be stained with various dyes to highlight different cells for further isolation. The most common laser microdissection methods are the laser catapult [8, 23] and the laser capture [24, 25] techniques. In the catapult system, a focused ultraviolet (UV) laser is used to burn or ablate an area around the cell(s) of interest on a tissue section contained on a glass slide. The UV laser beam is small enough that it can ablate an area around a single cell. The laser is then defocused and positioned directly below the cell of interest. The cell is then "catapulted" off the slide into a collecting cap via light pressure from the defocused beam. In the laser capture method, an infrared laser beam is positioned above the tissue section that is sandwiched between a plastic film and glass slide. When the laser beam is focused on a cell of interest, the plastic melts and penetrates the area around the cell. When the beam is turned off, the plastic solidifies and sticks to the cell, which can then be removed or

captured from the tissue section. The cells removed by both the laser capture and catapult systems can be stored or used immediately for the isolation of RNA, DNA, or protein. There are several reviews discussing methods and applications of LCM. [26-28]

Recently developed technique is Laser Scanning Cytometry (LSM) which allows imaging and quantitative analysis of individual cells in tissues *in situ*. [29] This technique can provide information on cellular responses that occur within physiological tissue microenvironments.

Most microarray studies using laser microdissection techniques utilize hundreds to thousands of cells for labeling purposes as the amount of RNA from a single cell is not sufficient for probe reduction. Several strategies to overcome this problem have been developed - for example PCR amplification method, which requires only picogram level of RNA for detection.

2.1.4. Microfluidics/microarray devices

Because of the small reaction volumes and the ability for automation, microfluidic devices are the ideal platform to analyze single cells in a high-throughput, highly parallel fashion. This method allows analyzing thousands of cells on a single device simultaneously.

Excellent review on microwell devices was recently published by Lindstrom and Andersson-Svahn. [30] This review is focused on the microwells that are used in single cell analysis. In this work, the authors cover different microwell device properties, such

as the well size, shape, [31-34] number of wells, and materials [34-36] for microwells preparation. The authors also described difficulties in different types of cells separation, possibility of dynamic cell monitoring and detailed example of single cell analysis in biological assays. Several examples of the well morphology are shown in the Figure **1**.

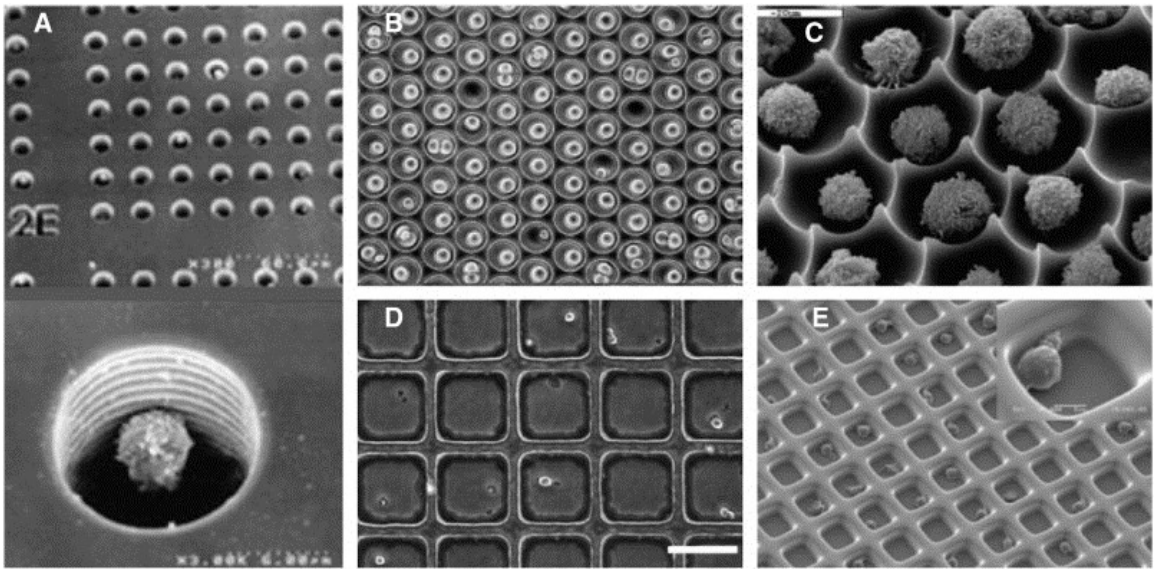


Figure 1. A set of microwell approaches, described in detail in (A) (Tokimitsu et al. [34]) close up of one well with a diameter of 10 μm, (B) (Rettig and Folch [32]) d = 25 μm, (C) (Deutsch et al. [35]) d = 20 μm, (D) (Chin et al. [31]) side = 100 μm, and (E) (Revzin et al. [33]) close up of one well with a side of 15 μm.

Another review of the same group of authors was focused on the microdevices classification and overview. [37] This work describes different approaches for isolation

of single cells using microwells [30], patterns [28, 38, 39], droplets (Figure 2) [40-43] and different kind of traps. Traps were classified as mechanical, [44] magnetic, [45] hydrodynamic, [46] optical, [47] dielectrophoretic, [48] and acoustic traps. [49]

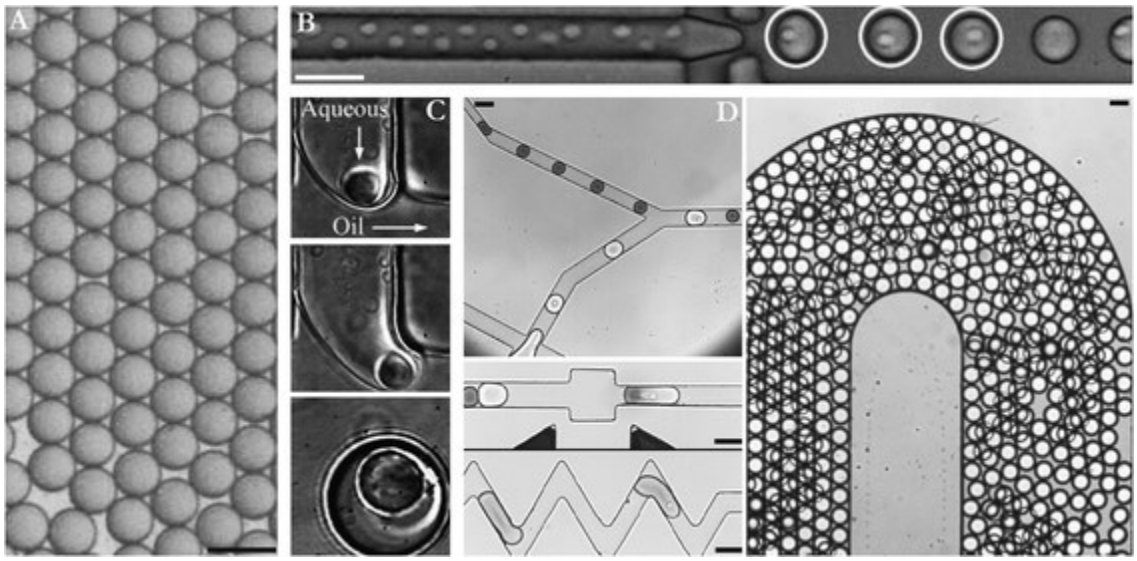


Figure 2. A set of droplet approaches that are discussed in this article and described in detail in: (A) ref. [43], (B) ref. [41] and (C) ref. [42], cell -size: 10 μm; and (D) ref. [40]. Scale bars 50 μm.

Hong J.W. et al. showed that these microfluidics devices could be used to isolate mRNA and DNA from a small number of bacterial or mammalian cells. [50] Other work by Marcus J.S. et al. discusses the ability of microfluidic assay to implement all five steps

(cell capture, cell lysis, mRNA purification, cDNA synthesis, cDNA purification) on one integrated device. [51] The scheme of this device is shown on the Figure **3**.

In this paper, the authors presented a microfluidic device that could be used for extraction of genetic material from a single cell. Using PCR they were able to detect several thousands of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene copies from a single NIH 3T3 fibroblast.

Recent work by Wang X. et al. [52] introduced a combination of microfluidic device with optical tweezers for sorting of small cell populations with high accuracy. For target cell recognition, the authors developed an image processing methodology, which allows recognition of cell features and fluorescence. Target cells are then moved by optical tweezers to desired area. The advantages of this method are the high recovery rate and purity in small populations of cells.

An interesting technique was developed by Phillips K. et al. [53] in which cells were continuously flowing through the microfluidic chip and were lysed by pulsing laser simultaneously. An applied electric field was used for the separation of cell membranes with fluorescent labels. The authors achieved processing of 300 cells/hr using the presented device, while the standard rate for isolation in microfluidic devices is on the level of approximately 100 cells/hr. [54]

Recently Lin et al. developed a novel microfabrication free device for single cells isolation, introducing Microscale Oil-Covered Cell Array (MOCCA). [55] In this work,

the authors used plasma treatment of masked conventional 25x25 mm glass slides to obtain 4000 circular (250 μm in diameter) hydrophilic areas. Obtained glass slide was submerged into cell medium solution that contains different concentrations of *E. coli* and then carefully washed with mineral oil. Hydrophilic discs on the surface trap small amount of cell medium with isolated cells. Procedure of cells isolation is shown in Figure 4. Using this method, the authors obtained a standard Poisson distribution of cells in droplets on a glass slide with the average of 2.23 cells per droplet. This method allows separating approximately 3000 single cells in about 10 minutes, and the cost of preparation is relatively cheap, compare to conventional microfluidic devices.

However, microfluidics devices have some disadvantages. This technology is still in the development stage and there are some complications appear under micro-scale fluidic conditions. Capillary forces, surface roughness and chemical interactions of construction materials become more dominant at the micro-scale; sometimes the processes in microfluidic devices are more complicated than in the standard laboratory environment. The most important disadvantages of microfluidic devices are high signal-to-noise ratio, and lack of precisely formulated geometry of the channels.

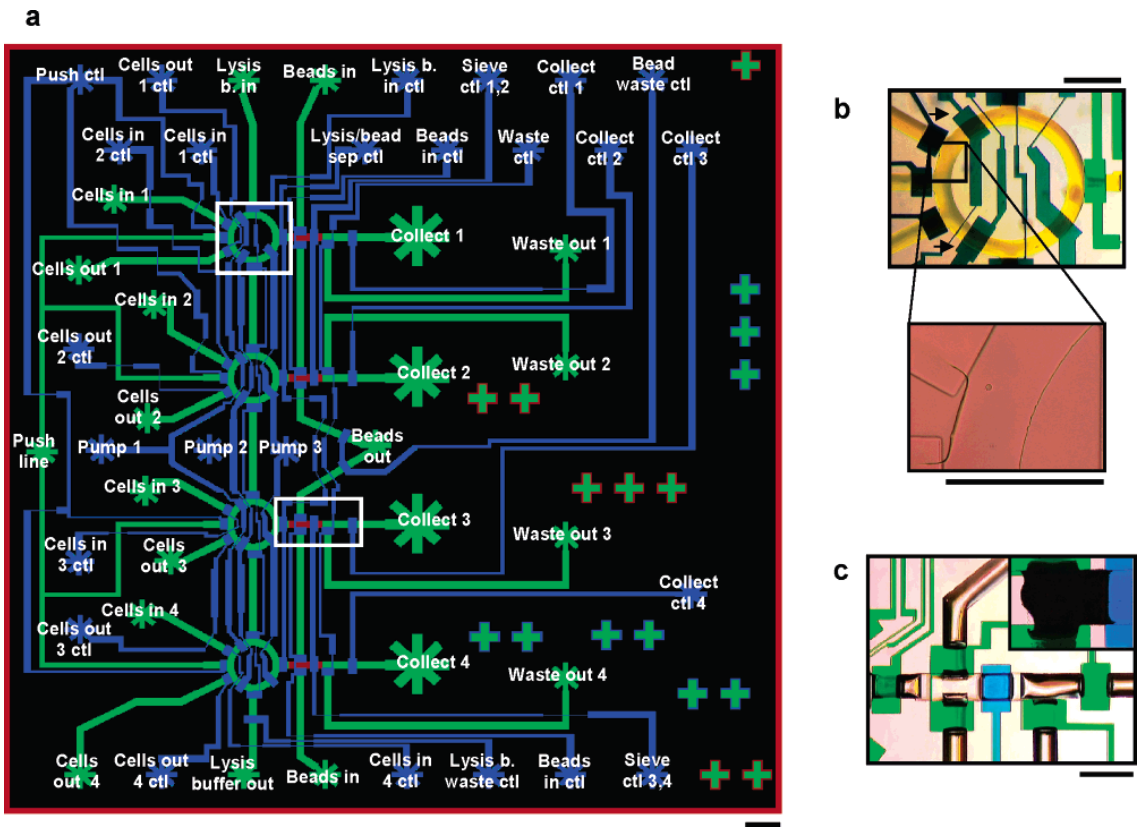


Figure 3. 4plex mRNA isolation/first strand synthesis device. (a) AutoCAD drawing of a device with inputs and outputs labeled according to function. Rounded flow channels are depicted in green and control channels are shown in blue. Unrounded (rectangular profile) flow channels for affinity column construction are shown in red. Portions of drawing in white boxes are shown in (b) and (c), respectively. (b) Optical micrographs of the lysis ring and an NIH/3T3 cell captured in the ring. (c) Optical micrographs of the affinity column construction area and a stacked column. Scale bars are 400 μm. (Reproduced from [51]).

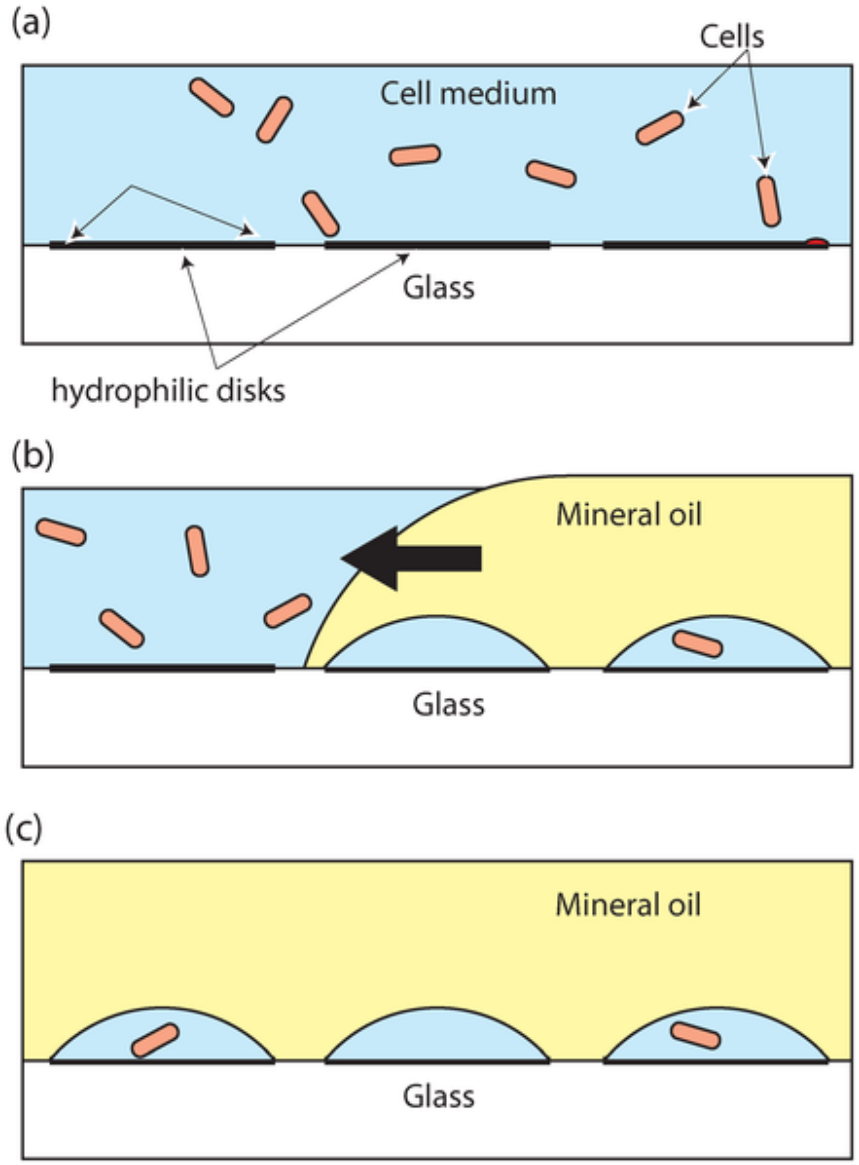


Figure 4. (a) Flood the microscale plasma activated template-patterned cover glass with cell medium; (b) Flood the cover glass using mineral oil to repel cell medium. Small volumes of medium still occupy hydrophilic disks on the treated surface; (c) After completion, some droplets in the array contain cells.

2.2. SINGLE CELL ANALYSIS METHODS

Single cell analysis methods are usually based on the detection of nucleic acids, protein expression levels and change of the surface potential. This part of review will highlight the most common methods for single cell analysis.

2.2.1. Fluorescence-based methods

2.2.1.1. Förster resonance energy transfer (FRET)

FRET is a distance-dependent method for detecting binding interactions. Fluorescent signals are generated or quenched based on the spatial separation of two chromophores. An energy transfer couple consists of a donor fluorophore and an acceptor chromophore. When FRET conditions are met (e.g. the donor and acceptor are in proximity), excitation energy is transferred from donor to acceptor using a non-radiative energy transfer mechanism, returning the fluorophore to its ground state without emission (Figure **5**).

When the two species are separated, the donor generates its characteristic emission spectrum, unaffected by the acceptor. The two most important conditions for FRET are the distance between donor and acceptor and the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. There are two types of acceptors. Quenchers are acceptors that are not fluorescent and therefore, cause the donor simply to decrease its fluorescence emission intensity.

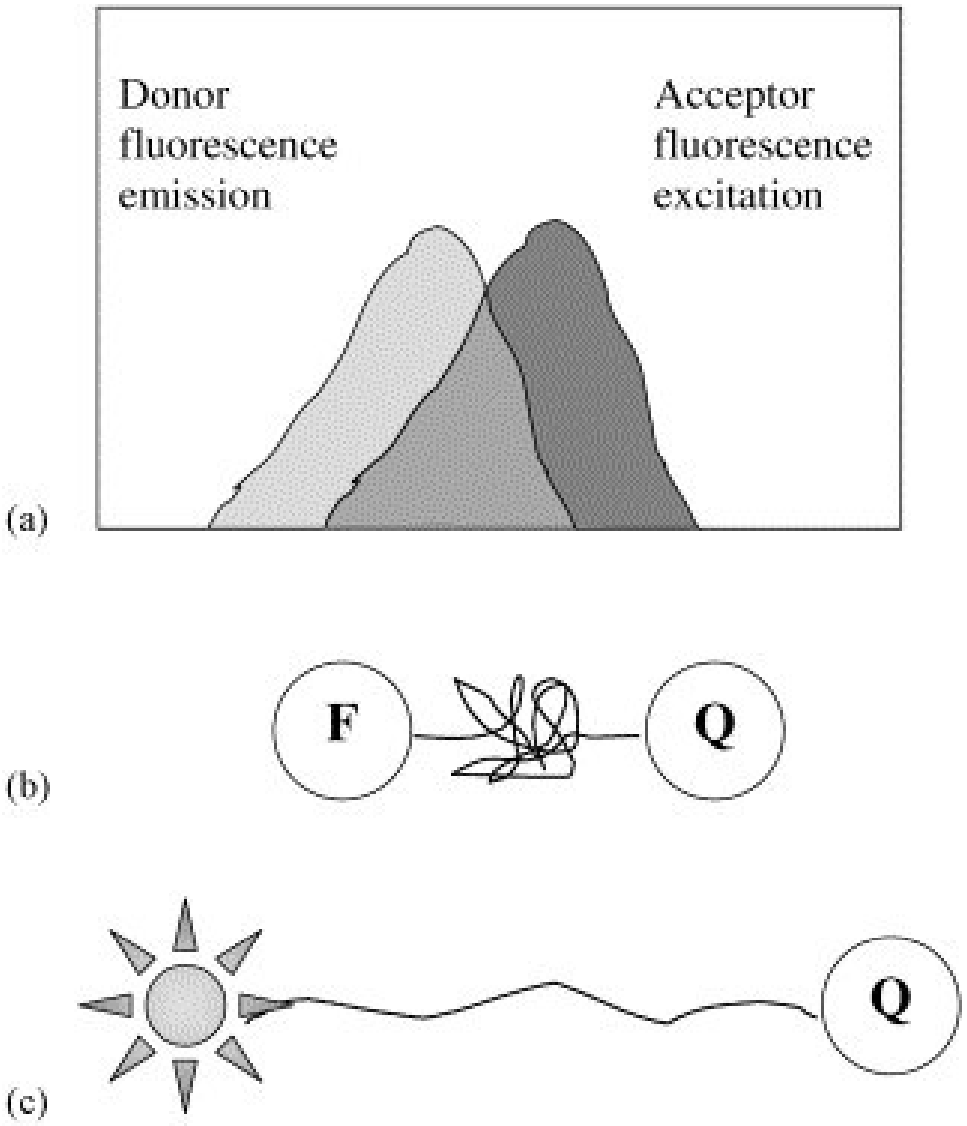


Figure 5. The FRET concept. (a) The overlapping spectra of the donor emission and the acceptor excitation, (b) with donor (F) and quencher (Q) in proximity, no fluorescent signal is generated, but (c) when separated, the donor is able to emit fluorescence uninhibited.

Acceptors can also be fluorescent dyes that accept the energy non-radiatively from the donor and then re-emit the energy with its own characteristic emission spectrum. In this form of FRET, donor fluorescence decreases at the expense of an increase in acceptor fluorescence. In addition to the two criteria mention above, the donor and acceptor orientations must have overlapping transition dipoles for effective quenching. The distance effect is proportional to R^{-6}, where R is the intermolecular separation distance between donor and acceptor. This distance effect in FRET is ideal for detecting biological macromolecule interactions and conformational changes occurring on the order of 10–100 Å.

FRET technique could be used for quantification of proteins expression. Two co-expressed proteins were found to keep the same expression ratio in several single cells with slight cell-to-cell variability. [56] This method is very important for the development of FRET based sensors.

Although FRET is a sensitive method for determining spatial separation or distance changes, it has several limitations. In ensembles of molecules, the signal provides only an average distance and gives no information as to which species has moved. FRET can be affected by the presence of other dyes that can act as donors or acceptors, therefore having excessive noise. The FRET mechanism has been incorporated into several detection designs, including molecular beacons [57-59] and PCR product detection assays. [60]

2.2.1.2. Fluorescence in Situ Hybridization (FISH)

Another method for determining the genes expression pattern is using FISH technique, which allows one to analyze expression of genes without destroying cell. [61, 62] This method is used to detect presence or absence of specific DNA sequences in the cells by using complimentary strands of fluorescently-labeled oligonucleotide probes. The fluorescent-labeled probes are detected using standard microscopes, laser confocal systems or charge-coupled device (CCD) cameras. Multiple fluorescent species can be simultaneously detected using multiple fluorescent labels in conjunction with wavelength filter systems. Computerized imaging advances and detection systems with enhanced sensitivity has allowed FISH to become a routine method for cellular DNA and RNA detection.

Modification of this method called RNA-FISH was used for detection of mRNA molecules in single cells by attaching labeled oligonucleotide to the targeted molecules of genes of interest. [63] This method allows direct quantification of mRNA expression by measuring the fluorescent intensity and could be used for comparison of genes expression from different single cells. Sensitivity of this method allows detecting up to single molecule of mRNA in a single cell. [64] For example, single molecule FISH microscopy has been used to assess the isoform variability for the genes CAPRIN1 and MKNK2 in HeLa and Rpe1 cells showing large cell to cell heterogeneity in isoform ratios. [64, 65]

FISH method can detect allelic variations, single nucleotide polymorphisms or the presence of genetic defects. However this approach is limited in its ability to provide accurate information about the entire mRNA population of the cell. [66]

2.2.1.3. Fluorescent nanoparticles

One of the most powerful tools in research for different molecules detection is fluorescent nanoparticles. Nanoparticles have been coupled to biomolecules [67] and microspheres [68] for fluorescence-based detection assays. [69, 70] Fluorescent nanoparticles for DNA detection consist of polymer nanospheres [70, 71] and quantum dots. [67, 68, 72] DNA-coupled latex nanoparticles are embedded with dyes and provide fluorescent emissions with a brighter signal and greater resistance to photo bleaching than organic fluorophores. [70]

Nie and co-workers employed 20 nm diameter latex nanobeads coupled to DNA-binding proteins as probe elements. [70] DNA-binding proteins, such as restriction enzymes, were rendered inactive in the absence of correct metal ion cofactors. The specific DNA sequences recognized by the restriction enzyme were used as the target sequences. The inactive enzyme–nanoparticle cluster was hybridized to DNA and viewed with standard microscopy techniques. The bacteriophage lamda genome, which contains multiple copies of the target DNA sequence, was correctly identified over other phage DNA. Although nanoparticle detection schemes provide reasonable detection capabilities, nanoparticles are larger than typical organic fluorophores and may, therefore, influence binding kinetics and give rise to steric hindrance problems. [70]

A multifunctional nanoprobe capable of targeting glioma cells, detectable by both magnetic resonance imaging and fluorescence microscopy, was developed by Zhang and co-workers. [73] In this study, iron oxide nanoparticles were coated by polyethylene glycol (PEG) linker with near-infrared (NIR) molecules for delivery inside cancer cells. Possibility of treating single cancer cells was discussed in the article. Figure 6 represents the delivery of nanoparticles to the 9L cells.

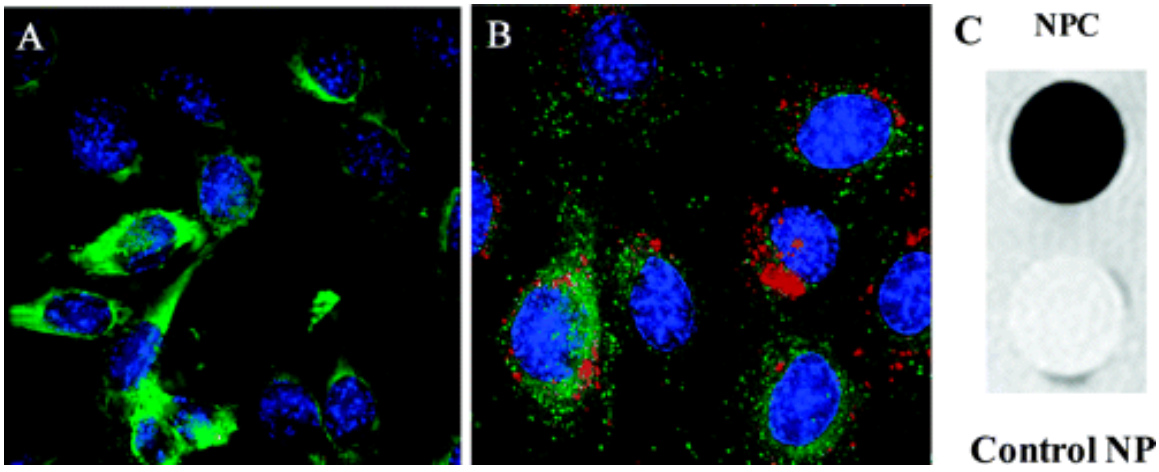


Figure 6.Confocal fluorescent images of 9L cells cultured with (A) control NP−Cy5.5 and (B) NPC−Cy5.5. (C) MR phantom image of 9L cells cultured with NPCs and control NPs and embedded in agarose (4.7 T, spin−echo pulse sequence, TR 3000 ms, TE 15 ms).

Luminescent semiconductor quantum dot nanoparticles (for example, zinc sulfide capped with cadmium selenide) can be coupled to oligonucleotide probes and provide 20-fold higher brightness with 100-fold stability to photo bleaching over organic dyes. [67] Quantum dot emission bands depend on particle size [74] and can have one-third the spectral line width of organic dyes. [67] A significant advantage of these particles is that they can be excited with white light, making the excitation source a trivial component of the detection system.

Although not fluorescence-based, gold nanoparticle-based detection schemes [69] also incorporate optical methods and have shown promise in nucleic acid detection. Work by Mirkin and co-workers have allowed optical array-based gold nanoparticle detection methods for use with conventional flatbed scanners. [69] The detection method entails a surface-bound probe sequence along with a second oligonucleotide probe covalently attached to gold nanoparticles. [75] The target hybridization process involves a sandwich assay consisting of the surface-bound probe element, the target sequence and nanoparticle probe. Upon target hybridization, the gold nanoparticles form a polymer-like network, initiating a detectable color change.

Although fluorescent nanoparticles have a great potential for diagnostics and detection of single molecules, they often lack specificity of binding to desired sites. Most of nanoparticles or their products of degradation were found to be toxic for cells at high concentrations.

2.2.1.4. RT-PCR

Currently, the polymerase chain reaction (PCR) is the most widely used method to determine the expression of different genes in single cells. The PCR process combines the denatured target sequence with a heat-stable polymerase, deoxynucleotides and primer sequences. Primer sequences flank the target sequence, directing polymerase activity towards target amplification. By cycling between the polymerase reaction and target duplex denaturation, an exponential increase of target concentration occurs. The heat-stable polymerase eliminates the need for addition of fresh polymerase at each cycle. Fluorescent primers or fluorescent nucleotides can be used to create fluorescently-labeled amplified target sequences. If only one primer sequence is incorporated into the PCR, a linear amplification results, while two antiparallel primer sequences provide an exponential increase in target concentration.

While PCR techniques have dramatically impacted modern genomics, there are some common problems associated with PCR. Some heat-stable polymerases are often error-prone, causing undesired changes in the amplified sequence. These errors can occur in the amplified product through incorrect base pairing or improper primer binding. PCR is also susceptible to contamination and can amplify unintended DNA from airborne microorganisms or human skin cells.

For this purpose, several modifications could be made to increase sensitivity of the method: total reaction volumes should be decreased to prevent loss of signal due to

low concentration, the reactions of reverse transcription and PCR should be merged in single tube to avoid loss of material due to multi-tube procedure.

The most sensitive method for genes detection from low population of cells is the real time quantitative PCR (RT-qPCR) [76-78] which usually allows to extract genes from 1-50 cells. However, qRT-PCR detection of even high-abundance transcripts from a minute number of cells is challenging, as evidenced by high cycle threshold (CT) values and a lack of consistency between replicates. Often low- and medium-abundance transcripts are not detected at all. There are several boosting techniques that allow amplification of the fluorescence signal in PCR reactions are currently on the market, one of them is MessageBooster™ from Epicentre Biotechnologies (Figure **7**).

The use of modification methods for the PCR analysis allows to increase sensitivity and to apply this method to determine genes expression from a single cell. Single cell PCR analysis is beneficial to the conventional method because there is no need to purify the genetic material from cell debris, proteins and salts. Thus, it significantly reduces the time between the cell isolation and analysis compare to the conventional PCR method.

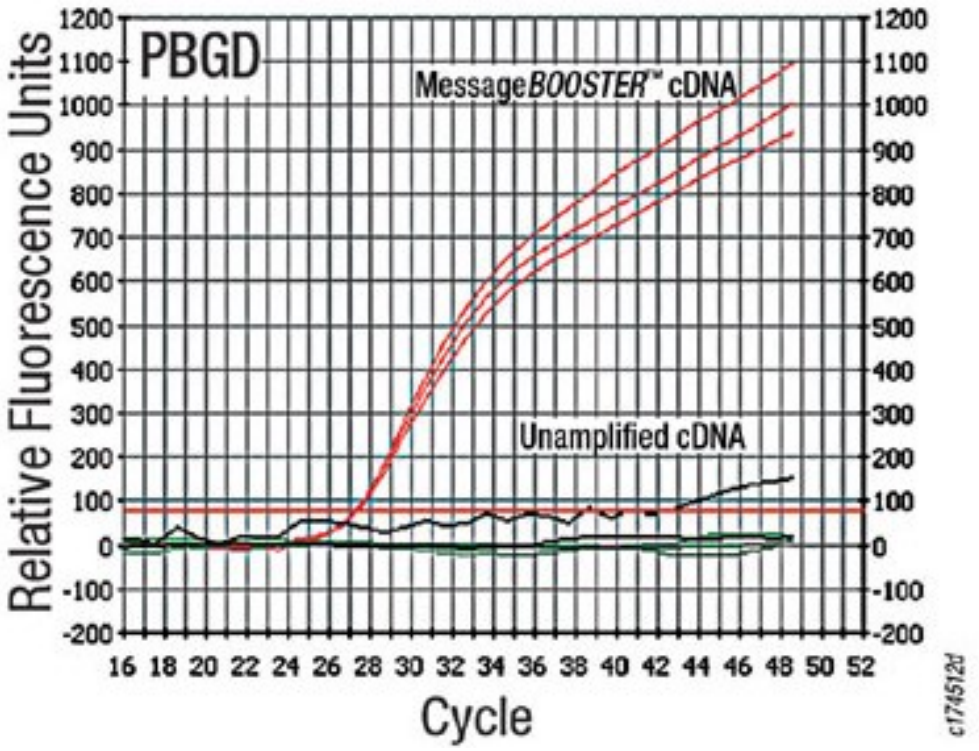


Figure 7. cDNA produced from 10 pg of total RNA using MessageBooster™ cDNA Synthesis Kit for qPCR significantly improves the sensitivity of detection of a low-abundance transcript compared to cDNA produced from 10pg of unamplified RNA. (Reproduced from [56])

2.2.2. Fiber-optic probes

One of the novel methods for single cell analysis which allows monitoring cell properties is the fiber-optic probe. In recent years, a key stimulus for the development of optical biosensors has been the availability of high-quality fibers and optoelectronic components at a reasonable cost. The advantages of optical biosensors are their speed, the

immunity of the signal to electrical or magnetic interference and the potential for higher information content (spectrum of information available). Optical methods are readily multiplexed; samples can be interrogated with many wavelengths simultaneously without interfering with one another. The optical biosensor format may involve direct detection of the target of interest or indirect detection through optically labeled probes.

The optical transducer may detect changes in the absorbance, fluorescence/phosphorescence, chemiluminescence, reflectance, light scattering or refractive index. A large variety of optical methods have been used in biosensors, however, those devices based on fluorescence spectroscopy, surface plasmon resonance, interferometry and spectroscopy of guided modes in optical waveguide structures (grating coupler and resonant mirror) are the most common. [79, 80] In practice, fiber-optics can be coupled with all optical techniques, thus increasing their versatility.

Optical fiber-based endoscope for a single cell was designed by Yan, R et al. [11] In this work, the authors have shown the ability of the manufactured endoscope to penetrate the cell wall and detect optical signals from subcellular regions with high spatial resolution. The ability of the endoscope to deliver cargo such as quantum dots inside the cells was shown. The scheme of the fiber-based endoscope is shown in Figure **8**. Researchers also shown, that the endoscope can be used for the detection of pH inside droplet of liquid using the pH sensitive dye *N*-fluorescein acrylamide (FLAC).

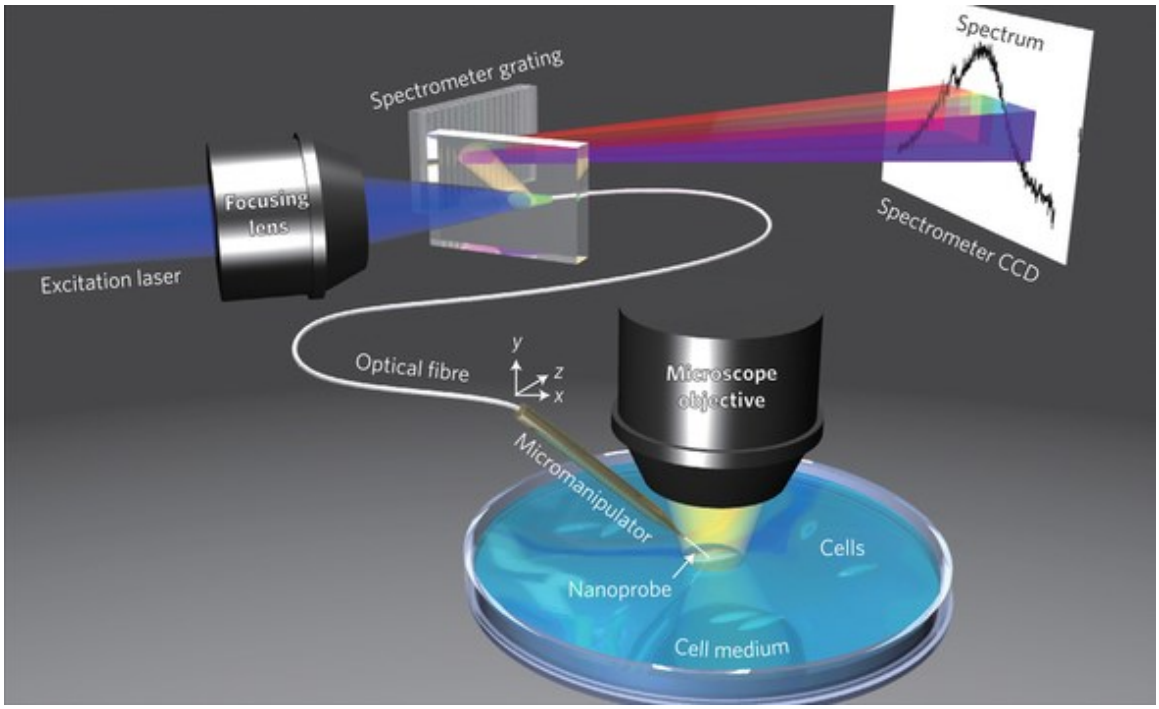


Figure 8. Schematic illustration of the nanowire-based cell endoscope system. The nanowire endoscope, consisting of a nanowire waveguide fixed on the tapered tip of an optical fiber, can be inserted into a single living cell at designated positions using a three-axis micromanipulating system for spot delivery of payloads. The nanowire endoscope can be optically coupled to either an excitation laser to function as a local light source for subcellular imaging or a spectrometer to collect local optical signals. CCD - charge-coupled device.

The nanoscale size of these new classes of sensors allows measurements in individual cells. This provides opportunities for *in vivo* monitoring of processes within live cells. Cullum et al. [81] used optical fibers with a distal-end diameter of less than 1 μm, coated with antibodies, to detect the presence of toxic chemicals within a single cell. They were able to measure the concentration of benzopyrene tetrol (BPT) within human mammary carcinoma cells and rat liver epithelial cells. Vo-Dinh et al. fabricated nanoprobes with optical fibers pulled down to tips with the distal ends having sizes of approximately 30−50 nm. [82] Using these probes, it has become possible to probe chemical species at specific spots. Nano-controlled release systems have been devised for optical biosensing of peroxide concentration. [83]

The ability to interact with inner cell structure without damaging the cell introduces us to a new level of single cell analysis as live cell monitoring. Nano-sized fiber-optic probes modified with biomolecules for single cell monitoring are the biosensors. The chapter 2.3 discusses biosensors with emphasis on 1D structures that are used in biosensing part.

2.2.3. ELISA

Another method of analysis that could possess necessary sensitivity for single cell analysis is enzyme-linked immunosorbent assay, or ELISA. This method is one of the most commonly used techniques in research and clinical laboratories of all kinds. It can be used to detect a variety of molecules and proteins in the body including serum proteins, hormones, drugs, antibodies, as well as other antigens. [84-89] This method has

been successfully utilized in a number of medical screening tests, and for detection of pathological conditions. [90, 91] As is the case with any assay, this method of analysis has undergone numerous enhancements since it was first described in the 1960s.

ELISA uses the specific ligands present in the body as a basis for a colorimetric assay to determine whether or not a particular antigen is present. This technique works by using antibodies which bind to the specific antigen and introducing to the system specific enzyme capable of producing a colorimetric signal that could report the presence of the antigen of interest. As ELISAs have evolved, several different variations of the technique have emerged. [29] There are four different common variations in the ELISA method: the simplest one is direct method, then indirect, and sandwich ELISA which could be direct or indirect (Figure 9Figure 9. Various common types of ELISA based assay.). Direct ELISA is based on the detection of antigen attached to the surface of well plates with the primary antibody conjugated with enzyme. Indirect method introduces secondary enzyme conjugated antibodies for the detection of primary antibodies that will react with immobilized antigens. In a sandwich ELISA assay, capture antibodies are immobilized on the surface for detection of specific antigens or specific antibodies in solutions. [92] After antigen immobilization, in the direct method, solution of another primary antibody from different species specific to the same antigen and conjugated with enzyme molecules is added. While in the indirect method for sandwich ELISA after incubation with primary antibodies from different species, secondary antibodies are added for detection of added primary antibodies.

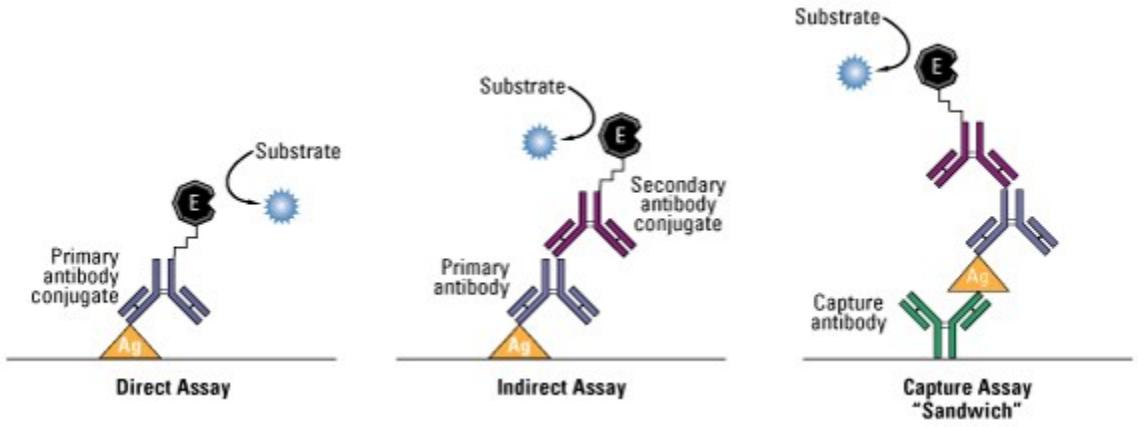


Figure 9. Various common types of ELISA based assay. [93]

ELISA is based on the detection of colorimetric product development from substrates that undergo transformation in the presence of peroxidase molecules. Color development reaction could be monitored over time in the kinetic study or just taking an end-point reading at the specific time point. This method can provide qualitative as well as quantitative data for analysis of presence of specific antigen molecules.

Conventional ELISA that is performed in standard well plates has many advantages and certain disadvantages. Numerous improvements have been successfully developed to increase sensitivity of this method. [94] The main advantage of this method is that it provides excellent sensitivity and is used for detection of analyte in solutions of extremely low concentration. On average this technique is capable of accurate measurements down to 1-10 fmol per well [95] and is sensitive enough to detect subtle

differences in proteins and molecules. For example, ELISA is capable of distinguishing IgG antibodies with different affinities. This level of specificity with such high sensitivity makes ELISA an extremely valuable and versatile evaluation technique.

Recent development in the manufacturing of portable microfluidic ELISA based assay devices was shown in the works done by the Whiteside's group from Harvard University. [96-98] Utilization of paper-based devices could lower costs for the ELISA analysis and their portable size will improve the convenience in the performance of the assay. They also discuss that the detection of colorimetric signal produced during the analysis could be performed using digital camera or desktop scanner, which will further reduce the cost and complexity of the conventional ELISA. However, authors report that the sensitivity of manufactured devices will be one order of magnitude lower than that for the conventional method.

Overall, disadvantages for ELISA include expensive equipment and reagents and a long time to perform the analysis. The most prohibitive of which is the extensive temporal commitment required to perform an ELISA. Moreover, low sensitivity limits the efficacy of some ELISAs and an increase in sensitivity is necessary. One example of a need for increased sensitivity is in the human immunodeficiency virus (HIV) ELISA because this method sometimes has a false positive results incidence. An increase in sensitivity could limit this false positives occurrence rate and provide more accurate results. It is also necessary step to implementation of the ELISA method towards the single cell analysis.

2.3. BIOSENSORS

A biosensor is an analytical device which integrates a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of analytes. [99-101] Typically, biosensors consist of three components: the detector, which identifies the signal, the transducer, which converts signal to recognizable format, and the output system, which amplifies and displays the output format of a signal. [102] In the general scheme of a biosensor, the biological recognition element responds to the target compound and the transducer converts the biological response to a detectable signal, which can be measured electrochemically, optically, acoustically, mechanically, calorimetrically, or electronically, and then correlated with the analyte concentration. [103]

2.3.1. Classification of biosensors

Biosensors can be classified by their bio-recognition system or depending on the method of signal transduction. The five main bio-recognition mechanisms used in biosensor technology are: antibody-antigen, [104] enzyme-substrate, [105] nucleic acids-complementary sequences, [106] cell based [106-108] and biomimetic materials based. [109] Each of the recognition mechanisms will be discussed below.

- Antibody-antigen sensing mechanism uses the high specificity of the reaction between antibodies and antigens to determine molecules of interest in the solution of interest. These biosensors should be used in conditions where other non-

specific interactions are minimized. The binding of an antigen to an antibody could be detected using fluorescent labeling or by change of refractive index of substrate where antibodies are immobilized. [105]

- Enzyme-based sensors utilize the catalytic activity of the enzymes immobilized on the substrate. Targeted molecules are processed by enzymatic reaction and products of this reaction could be easily detected directly or using an indicator. [105] This type of biosensor has the ability to detect much lower quantities of analyte than using the normal binding techniques.

- Biosensors based on nucleic acids use complimentary relationships between adenosine and thymine, cytosine and guanosine in DNA form. These sensors are capable of detection of trace amounts of complimentary DNA that binds to the known sequence in the sensor. The detection of DNA could be carried out utilizing the PCR reaction or, if the probe has a fluorescent label – it could be visualized under a microscope. [104]

- Sensors that use cells of microorganisms and viruses could be used to detect specific molecules or overall state of the surrounding environment. [106] These systems usually use cell viability, metabolism and bioluminescence as indicator of presence of analytes. However, the proteins expressed by cells could also be used as a bioreceptors for detection of specific molecules.

- Biomimetic biosensors are an artificial or synthetic sensor that mimics the function of a natural biosensor. These types of biosensors usually utilize aptamers in the detection mechanism and this type of biosensors usually called aptosensors. Aptamers are synthetic strands of DNA, specifically designed to recognize specific targets, such as amino acids, proteins oligosaccharides and other molecules. [109]

Biosensor transduction can be classified in four categories: optical detection, [104] electrochemical, [104] mass-sensitive [106] and thermal detection. [110]

- Biosensors based on the optical signal detection are the most diverse class in their family because they can be used for many types of spectroscopy, such as absorption, fluorescence, Raman spectroscopy, surface enhanced Raman spectroscopy (SERS), refraction and dispersion spectrometry. [104] These methods are capable of detecting different properties, such as energy, amplitude, polarization, decay time and phase. The most common measurement is amplitude, which can be easily correlated to the concentration of detectable analyte.

- Electrochemical biosensors measure the current change in the oxidation and reduction reactions that take place upon sensing targeted molecules. This current is then analyzed and correlated with the concentration of analyte of interest. [104]

- Biosensors that are based on the mass-sensitive measurements detect mass changes caused by chemical binding of analyte to small piezoelectric crystals. The

detection is based on the change of initial frequency of crystal oscillation, which depends on the mass of the crystal, and therefore the mass of analyte could be calculated. [106]

- Thermal biosensors measure the changes in temperature due to the reaction between enzyme molecules and a suitable analyte. [110] The change in temperature can be correlated to the amount of products formed in this reaction.

Biosensors could also be classified by the type of nanostructures that is used in manufacturing process. In this section the main attention will be focused on biosensors utilizing 1D nanomaterials.

2.3.2. 1D nanomaterials used in biosensors

Single dimensional nanomaterials that are currently widely investigated are nanowires, nanorods, nanoprobes and nanotubes. Several examples of nanoprobes for single cell piercing using optical fiber-based probes were discussed in the single cell analysis section.

Boron-doped silicon nanowires (SiNWs) were reported by Cui et al. [111] to create highly sensitive, real-time electrically based sensors for biological and chemical species. The amine and oxide-functionalized SiNWs exhibited pH-dependent conductance that was linear over a large dynamic range and due to the change in surface charge during protonation and deprotonation. Biotin-modified SiNWs were used to detect streptavidin down to at least a picomolar concentration range. The scheme of SiNW

modification and sensing mechanism is shown in Figure 10. In addition, antigen-functionalized SiNWs showed reversible antibody binding and concentration-dependent detection in real time. The small size and capability of these semiconductor nanowires for sensitive, label-free, real-time detection of a wide range of chemical and biological species can be exploited in array-based screening and *in vivo* diagnostics.

2.3.2.1. Tubular nanostructures

Tubular and other porous nanostructures were implemented in biosensors to increase the quantity and activity of the immobilized biomolecules. However, in view of their unique properties, these nanostructures provide opportunities for development of novel designs of biosensors. Several examples of tubular and other porous nanostructures in biosensors are shown below.

2.3.2.2. Carbon nanotubes

Since their discovery, carbon nanotubes have attracted great attention as nanoscale building blocks for microdevices. The nano-dimensions, graphitic surface chemistry and electronic properties of carbon nanotubes make them an ideal material for use in chemical and biochemical sensing. Both single-wall nanotubes (SWNT) and multiwall carbon nanotubes (MWNT) have been used in biosensors. [112, 113]

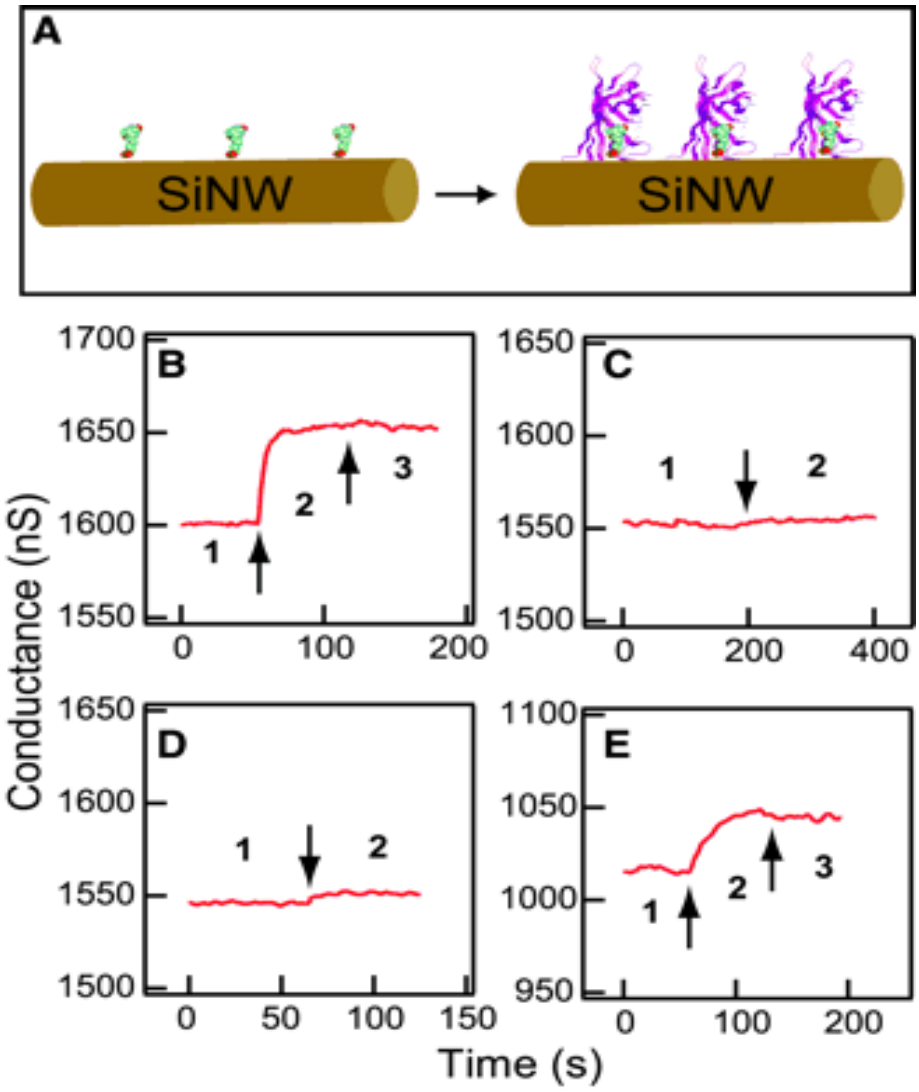


Figure 10. Real-time detection of protein binding. (A) Schematic illustrating a biotin-modified SiNW (left) and subsequent binding of streptavidin to the SiNW surface (right). The SiNW and streptavidin are drawn approximately to scale. (B) Plot of conductance versus time for a biotin-modified SiNW, where region 1 corresponds to buffer solution, region 2 corresponds to the addition of 250 nM streptavidin, and region 3 corresponds to pure buffer solution. (C) Conductance versus time for an unmodified SiNW; regions 1

and 2 are the same as in (B). (D) Conductance versus time for a biotin-modified SiNW, where region 1 corresponds to buffer solution and region 2 to the addition of a 250 nM streptavidin solution that was preincubated with 4 equivalents d-biotin. (E) Conductance versus time for a biotin-modified SiNW, where region 1 corresponds to buffer solution, region 2 corresponds to the addition of 25 pM streptavidin, and region 3 corresponds to pure buffer solution. Arrows mark the points when solutions were changed. (Reproduced from [111])

In one case, glucose oxidase was immobilized by coating onto the surface of single-wall nanotubes (SWNT) without a gross loss of enzyme activity. [114] The treatment of this bio-SWNT sensor with both a diffusive mediator and equilibrated glucose substrate enhanced the catalytic signal by more than one order of magnitude compared to that observed at an activated macro-carbon electrode. This enhanced performance was partly due to the high enzyme loading and partly because of better electrical communication ability of the nanotubes.

The direct electron transfer ability of carbon nanotubes has been exploited in other cases. For example, use of SWNT has made possible a direct electron transfer with the redox active centers of adsorbed oxidoreductase enzymes. [115] Both flavin adenine dinucleotide (FAD) and glucose oxidase (GOx) were found to spontaneously adsorb to unannealed carbon nanotubes that had been cast onto glassy carbon electrodes and to display quasi-reversible one-electron transfer. Similarly, GOx was found to

spontaneously adsorb to annealed, single-walled carbon nanotube paper and to display quasi-reversible one-electron transfer. In particular, GOx immobilized in this way was shown to maintain its substrate-specific enzyme activity in the presence of glucose.

Carbon nanotube-based electrochemiluminescence (ECL) biosensors have been described by Wohlstadter et al. [116] Several characteristics make carbon nanotubes useful for ECL-based assays. Firstly, they are conductive, can act as electrodes, and can generate ECL signal. Secondly, they can be functionalized for the immobilization of biomolecules. In addition, carbon nanotubes have a high surface area-to-weight ratio and most of this surface area is accessible to both electrochemical reactions and immobilization of biomolecules. However, many of these functions can be just as effectively fulfilled by other non-carbon nanostructures such as metallic nanoparticles or fibers. The procedure for fabrication carbon nanotube electrodes is shown on the Figure 11.

It is believed that the tubular fibrils become positioned within tunneling distance of the cofactors without too much denaturation of the enzyme. The combination of SWNT with redox active enzymes appears to offer a convenient platform for a fundamental understanding of biological redox reactions and the development of reagentless biosensors and nanobiosensors. Similarly, horseradish peroxidase adsorbed on a carbon nanotube microelectrode was found to transfer electrons directly to the electrode and retain its catalytic activity toward H_2O_2. [117]

Carbon nanotube array-based biosensors have also been reported. Aligned multiwall carbon nanotubes grown on platinum substrate have been described for the development of an amperometric biosensor. [113] The two array systems in this project were either acid treated, or air treated. The results showed that chemical etching was more effective in opening the carbon nanotubes and allowing the enzyme to enter the inner channel. It seems that the oxidation of the array introduced carboxylic groups at the open-ends, to provide a stabilizing hydrophilic environment that allowed for the adsorption and insertion of the enzyme into the cavity of the nanotubes. Also, the immobilization of the enzyme within nanotubes may permit a mediated direct electron transfer to the platinum substrate transducer.

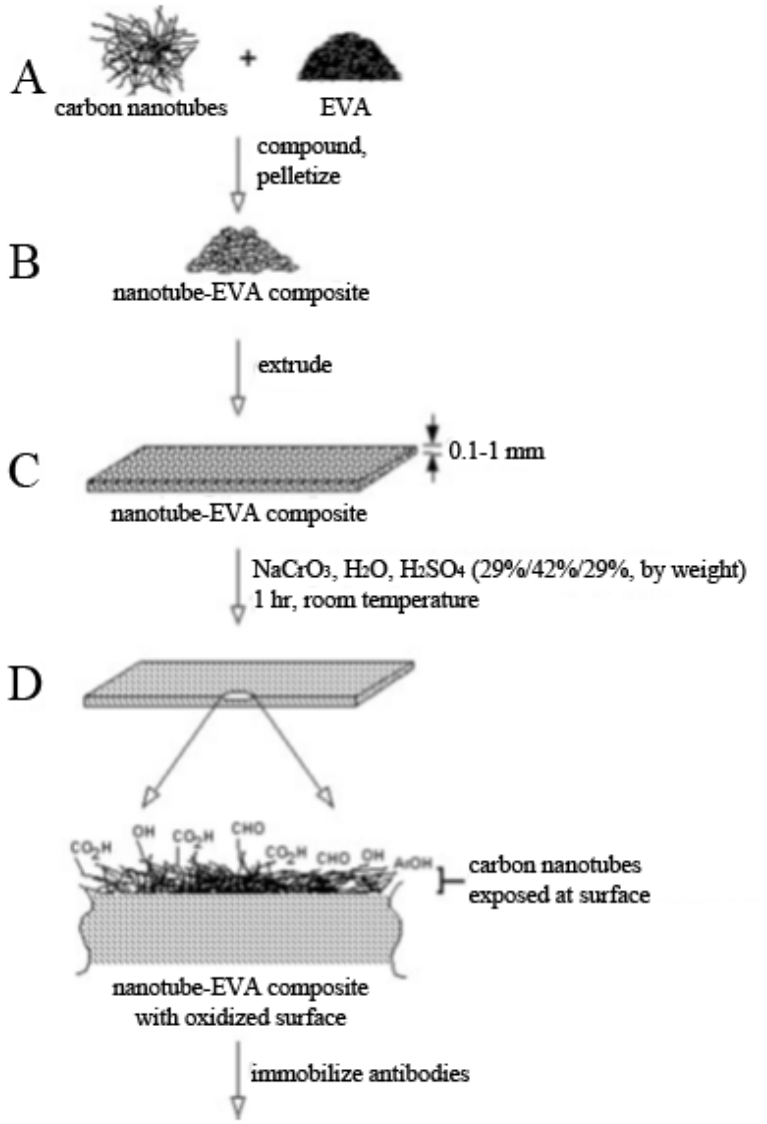


Figure 11. Procedure for fabricating nanotube-polymer electrodes. Compounding a mixture of EVA and carbon nanotubes (27 wt.-%) formed a composite that was extruded into sheets. Sheets of the extruded composite were floated on the surface of a chromis acid/sulfuric acid solution for approximately 1 h at room temperature, washed 4-5 times with deionized water, and allowed to dry in ambient air.

Recent work by Y. Gogotsi's group has shown the applicability of carbon nanotubes in the development of the sensor for a single cell analysis. [10] In this work, authors developed a probe, which consisted of glass capillary with multiwall carbon nanotube (length, 50–60 μm) attached to the tip. They have shown that this endoscope like device could be used for transport of liquids inside the cell to and from specific precise locations.

Carbon nanotubes have a great potential for use in biosensors, however, there are several disadvantages. These nanotubes are still costly to manufacture and very difficult to manipulate. Imperfections in processing and impurities in preparation of nanotubes lead to undesirable properties, and are difficult to overcome. There is also a concern that carbon nanotubes could be dangerous for health because they could build up in lungs or lead to cancer due to inhalation of minute amounts of these nanotubes.

2.3.2.3. Other nanotube materials

Arrays of nanoscopic gold tubes have been prepared by electrolysis deposition of the metal within the pores of polycarbonate particle track-etched membranes. [116] Glucose oxidase was immobilized onto the preformed self-assembled monolayers (SAMs) (mercaptoethylamine or mercaptopropionic acid) of gold tubes, via cross-linking with glutaraldehyde or covalent attachment by carbodiimide coupling. Glucose responses as large as 400 nA/(mM cm^2) were obtained. Miao Y.Q. et al. [118] proposed making biosensor using the same technique by immobilization of glucose oxidase on the polypyrrole nanotubes. Compared to conventional techniques, this immobilization

strategy enhanced the amount of the enzyme immobilized, the retention of the immobilized activity and the sensitivity of the biosensor.

Overall, single dimension nanostructures were shown to increase the sensitivity when they were incorporated in the biosensors as a substrate for biomolecules immobilization. Along with nanorods and nanotubes there is a possibility to use nanofibers that could be obtained by various methods including conventional extrusion, force spinning and recently gained attention electrospinning technique. This technique offers versatile and convenient method for fibers manufacturing and their properties control. Compare to the preparation of other 1D nanostructures, electrospinning method is pretty simple and could reduce the costs associated with biosensors production and implementation.

2.4. CONCLUDING REMARKS

Concluding the review, there are many potentially useful tools for single cell extraction and analysis. However, all the methods described above have their own advantages and disadvantages. Thus, there is a need for development of new methods, which will utilize the advantages of existing methods and potentially lower drawbacks. Potential solution for the new method is the combining the single cell extraction and analysis in a single device. Therefore we suggest using polymeric fiber-based devices for probing of single cells and conducting on-fiber PCR analysis for extracted genes.

In this work, research is going to be focused on designing of a novel type of biosensor – nanoporous microfiber-based device that could extract genetic material from individual cells. Microfibers, which possess extremely high surface area, would be most suitable for adsorbing high amount of proteins and other biomolecules from small amount of testing liquid. Compare to the other methods that have been described in the review, this system have a number of advantages: fibers are easy to manipulate, they are relatively cheap in production and they possess high sensitivity. Modification of these fibers with biomolecules will make them suitable for targeting of specific analytes such as DNA, RNA or proteins.

Different fiber materials with different characteristics (porosity, surface area, method of preparation) will be tested. Then fibers will be modified with biotin-oligo dT_{25} for targeting specifically mRNA molecules with poly-A tails. Extracted mRNA molecules will be analyzed by RT-PCR method. First, ability of fibers to extract mRNA

from cell lysates made of high amount of cells will be shown. Second, the sensitivity level of fiber-based devices for extraction of genetic material will be determined by conducting the experiment with diluted cell lysates. Finally, these probes will be used to extract genetic material from a living cell *in vitro*.

As a model system we will use vascular smooth muscle cells (VSMC), which have two different phenotypes – synthetic and contractile. Cells with the synthetic phenotype are softer, do not have a defined shape and have a high proliferation rate. Contractile cells are stiff, have a spindle-like shape and do not proliferate. This model is clinically important because transition from the contractile to synthetic phenotype is associated with restenosis. The two phenotypes show different levels of different gene expression. The contractile phenotype has higher expression levels of other cytoskeleton proteins such as γ-actin, β-actin, and smooth muscle myosin heavy chain compared to the synthetic phenotype.[82] Based on this difference, we will use this cell model to show that fiber-based mRNA extraction devices could be used for quantitative analysis of gene expression.

Analysis of proteins in the solutions will be analyzed by using the fiber-based devices for the ELISA method. Microfiber probes will be covered by antibodies specific to particular secondary antibody and tested for detection of the analyte. Sensitivity of the method will be compared to that of standard well-plate based method.

CHAPTER 3

SPECIFIC AIMS AND SIGNIFICANCE

3.1. OVERVIEW

Single cell analysis is a rapidly developing area with a number of potential applications in various fields. Single cell analysis allows for the gathering of information from individual cells as well as analysis of cell-to-cell variations in whole populations.[119] Analyzing genetic material in individual cells could reveal information that cannot be obtained from analysis of populations, one application of such information is in the treatment of diseases related to genetic dysfunction. Thus, single cell analysis is important in stem cell research, cancer detection and in studies of cell development processes. Currently all techniques for single cell gene analysis utilize the polymerase chain reaction (PCR)[78] technique, sometimes with pre-amplification step.[120]

Development of miniature microfluidic PCR devices has become one of the main focus areas of this research because their smaller size and specially designed architecture allow for faster PCR analysis.[121] However, PCR-on-a-chip microfluidics is rather expensive and its use requires even more rigorous training/expertise than in the case of conventional PCR. Furthermore, integration of the microfluidic PCR with sample preparation and detection of the products still remains a challenge.[121]

Therefore, a need exists to manufacture lower cost single cell analysis devices with the capability to analyze cells of the same sub-population. Even genetically similar

single cells could have different gene expression levels due to different states of growth and differentiation. These expression levels could also be affected by age, accumulation of mutations and epigenetic factors.

Recently, fiber-based microfluidic devices were proposed as inexpensive alternatives to on-chip microfluidic devices.[122] In fiber-based devices, liquid flow is controlled by capillary forces in microfiber or nanofiber yarns.[122, 123] In addition to improved manipulation of the liquid flow, fiber-based microfluidic devices also offer the advantages of simple fabrication and easy surface-modification which allow them to perform a variety of functions. One apparent advantage of PCR analysis performed on fiber-based devices is the possibility to collect the genetic material and process it on the same platform.

Here we propose to design a highly sensitive probing device for mRNA extraction from single cells. Extraction of mRNA will be performed using oligo dT_{25} molecules that will be conjugates to the fiber yarn due to the availability of poly A tails on most mRNA molecules. We will isolate mRNA from different tissue samples, cell culture lysates and from individual single cells to show a wide applicability of manufactured fibers. We will test several different methods for mRNA extraction and will compare them to the conventional method for mRNA extraction using two-step RT-PCR. We will also test modified fibers to detect specific secondary antibodies using a model fiber-based devices for the ELISA assay. Capture antibody will be immobilized on the fiber yarns surface and then exposed to the detection antibody (analyte) for further analysis.

3.2. SIGNIFICANCE

Single cell analysis provides researchers a new breakthrough in the understanding of single cell life cycle and could reveal the knowledge which analysis of cell populations cannot provide. Existing automated methods of single cell analysis have several disadvantages, which could be overcome by developing novel methods of analysis. There is an apparent need for novel sensitive and convenient experimental techniques for analysis of gene expression in individual cells. Our research is focused on the preparation of modified fiber-based devices for mRNA extraction from single cells. We expect that use of such devices will enable researchers to perform quick analysis of individual cells in complex systems, such as tissues comprising of several different cell types or cells that are being grown in co-culture. Such fiber sensors could possibly find an application in non-invasive biopsy, or in detection and recognition of a number of types of pathogenic bacteria. Another approach is focused on sensing antibody molecules in solutions that would allow one to detect specific antibodies in the blood or other body fluids of patients. These fiber-based devices could play a significant role in determination of various diseases such as HIV, hepatitis or detection of secondary antibodies related to drug addictions.

3.3. SPECIFIC AIM 1. PREPARATION AND CHARACTERIZATION OF OLIGO-DT_{25} MODIFIED FIBER YARNS

Previous research has shown that the [122] properties of electrospun fiber yarns can be successfully controlled and designed for specific applications. One could

manufacture and implement different kinds of probing devices based on these fibers, which are relatively cheap in preparation. They could also be adapted for application in biosensors. Here, we describe the preparation and characterization of microfluidic fiber-based devices with high specific surface area for analysis of genetic material. Several types of fibers were manufactured, characterized and modified with streptavidin and then with oligo dT_{25} complexes. Binding efficiency of streptavidin was assessed by two different methods of binding – covalent attachment and physical adsorption. Binding capacity of prepared fiber yarns was measured using fluorescently labeled oligo dT_{25} molecules. This study will also cover the investigation of surface morphology of the prepared fibers using SEM analysis during every step of treatment and washing.

3.4. SPECIFIC AIM 2. MRNA EXTRACTION FROM CELL LYSATES USING OLIGO-DT_{25} MODIFIED FIBER YARNS

The objective of this study was to test the ability of prepared fiber yarns to extract mRNA from cell lysates prepared from a large number of cells and determine optimal conditions for mRNA extraction. Sensitivity of fiber yarns in mRNA extraction experiments was investigated by the dilution of cell lysates down to the single cell level. This aim shows the applicability of fiber-based devices in the mRNA molecules extraction from solution of cell lysates. This aim also investigates the sensitivity of the method. The performance of the novel fiber-based devices described is evaluated in comparison with the commercially available kits for mRNA extraction. A pilot

experiment was also developed to perform mRNA extraction from the freshly excised tissues using fiber-based devices.

3.5. SPECIFIC AIM 3. DETECTION OF SECONDARY ANTIBODIES USING ANTIBODY MODIFIED PROBES

Application of modified fiber-based devices to the detection of antigen molecules was shown using enzyme-linked immune-sorbent assay (ELISA) method. ELISAs have found wide application in medical arena for detection of pathogens, drugs, and antibodies. It is also useful as a pre-screening method for detection of various diseases. Detection of proteins including antibodies could lead to the development of novel electrospun fiber yarns based biosensor capable of disease detection in an over the counter format. This at home detection could provide patients with an easy to use point of care device for early detection potentially leading to earlier care and more effective treatment. This aim describes the method of model secondary antibody detection using the novel modified protocol using fiber-based devices for ELISA. The developed method is compared to the standard method of analysis and the sensitivities of both methods were established. This comparison will highlight the advantages of the fiber-based devices for ELISA over traditional ELISAs.

CHAPTER 4

DEVELOPMENT AND CHARACTERIZATION OF FIBER-BASED DEVICES FOR mRNA EXTRACTION

Fiber yarns preparation by electrospinning method, investigation of yarn porosity and permeability was done by Chen-Chih Tsai from Dr. Kornev's group, collaborator from Materials Science Department at Clemson University. Modification of the yarns by streptavidin and further characterization was performed by the author in Dr. Vetregel's lab.

4.1 INTRODUCTION

Recent advances in preparation of fiber yarn bundles by the electrospinning method have made it possible to precisely control fiber parameters. These parameters include thickness, porosity, hydrophobic and hydrophilic properties in the desired manner. Composition of the yarns is highly customizable and can be performed by combining different polymers into the dope to achieve the desired set of properties. The simplicity of the fiber preparation by electrospinning has made them attractive for various applications including filtration[124], textile manufacturing[125], composites[126], catalysts[121, 127] and a number of other biomedical applications. In the biomedical field, these nano-fibers were shown to have great potential in tissue engineering[128], wound dressing systems[119, 129] and drug delivery.[129, 130]

One of the important applications of electrospun fibers is the use of prepared fibers in sensoric applications such as gas sensors[123], optical sensors[131] and biosensors. [120, 132, 133] Nanoporous fibers are beneficial in this application due to high specific surface area values, which allow more of the sensing elements to be introduced on their surface. The electrospun fiber bundles have found application in the detection of various molecules and ions in a variety of research areas. [134, 135] Successful use of electrospun nano-fibers for various applications is due to the set of unique properties and ease of fiber preparation. There is an extensive amount of work in the literature on fabrication and enhancement of properties of electrospun fibers leads to the development of the novel application of these fibers described herein.

Based on the existing literature, electrospun fiber-based devices have shown to increase sensitivity in various applications in different research fields. Electrospinning method allows controlling fiber yarns composition and properties and provide simple method of biosensors preparation. Biosensors that will utilize polymeric fiber yarns as a substrate for immobilization of biological sensing molecules could benefit from particular properties of prepared yarns.

The current chapter is focused on the preparation and systematic characterization of electrospun micro- or nano-fibers for application as biosensors. We have focused our research on the evaluation of the fiber's potential for extraction of certain mRNA molecules from lysed cells. We investigated physical properties of prepared fiber yarns

and possible mechanisms for surface modification of developed yarn. One of the possible modifications was introduction of polyT molecules on the fibers surface.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Cellulose acetate (CA, MW: ~37 kDa), polyacrylonitrile (PAN), poly(methyl methacrylate) (PMMA, MW: ~120 kDa) and polyethylene oxide (PEO, M_w=1,000 kDa) were purchased from Sigma-Aldrich Corporation. Dimethylacetamide (DMAc) was purchased from Alfa Aesar. Nylon yarn with thickness about 0.3 mm was purchased from Middleburg Yarn Company. The yarn (containing ~170 filaments with diameter of ~25 μm), had estimated surface area of 1385 cm^2/g. Streptavidin was purchased from Rockland Immunochemicals, Inc. Biotin-oligo dT_{25}, and primers for targeting and housekeeping genes were purchased from Integrated DNA Technologies, Inc. DNAse, RNAse and Protease free water, Tris-Ethylenediaminetetraacetate (EDTA) (TE) buffer, sodium chloride and sodium dodecyl sulfate (SDS) were purchased from Acros Organics. RNaseZap®, TURBO DNA-free™ Kit and RETROscript® Kit were purchased from Ambion, Inc. RNeasy Mini Kit and QuantiTect SYBR Green PCR Kit were purchased from QIAGEN, Inc. Poly A25-Fluorescein was purchased from Integrated DNA Technologies, Inc. All other chemicals were purchased from Sigma-Aldrich and used as received unless otherwise specified.

4.2.2. Dope preparation

Polymer solutions of different composition were prepared using 10 g of dimethylacetamide as the solvent. For preparation of CA/PMMA/PEO mixture, 1 g of CA, 0.4 g of PMMA and 0.2 g of PEO were used; for CA/PMMA mixture – 1 g of CA and 0.4 g of PMMA; for PAN/PMMA – 1 g of PAN and 0.6 g of PMMA; for CA/PAN mixture – 1 g of each CA and PAN. Obtained mixtures were stirred with magnetic bar at 55°C for two hours prior to yarn formation.

4.2.3. Yarn formation

The yarns were formed from electrospun microfibers as described previously.[122] The desired polymer mixture solution was placed in a 10 ml syringe. During electrospinning, the syringe was covered with a flexible heater (Watlow, EHG SL10) to maintain the solution temperature at 55°C. A syringe pump (New Era Pump System, NE-300) was used to control the flow rate at 0.4 ml/hr. A home-made rotating mandrel with four alumina bars separated from each other by 20 cm was used as a fiber collector.[122] A high-voltage power supply (Glassman High Voltage, Inc.) was connected to the syringe through a stainless-steel needle (Gauge 20, EXEL). The needle was placed 30 cm away (counting from the needle tip) from the nearest face of the collector. Schematic diagram or the fiber preparation assembly is shown on the Figure 12.

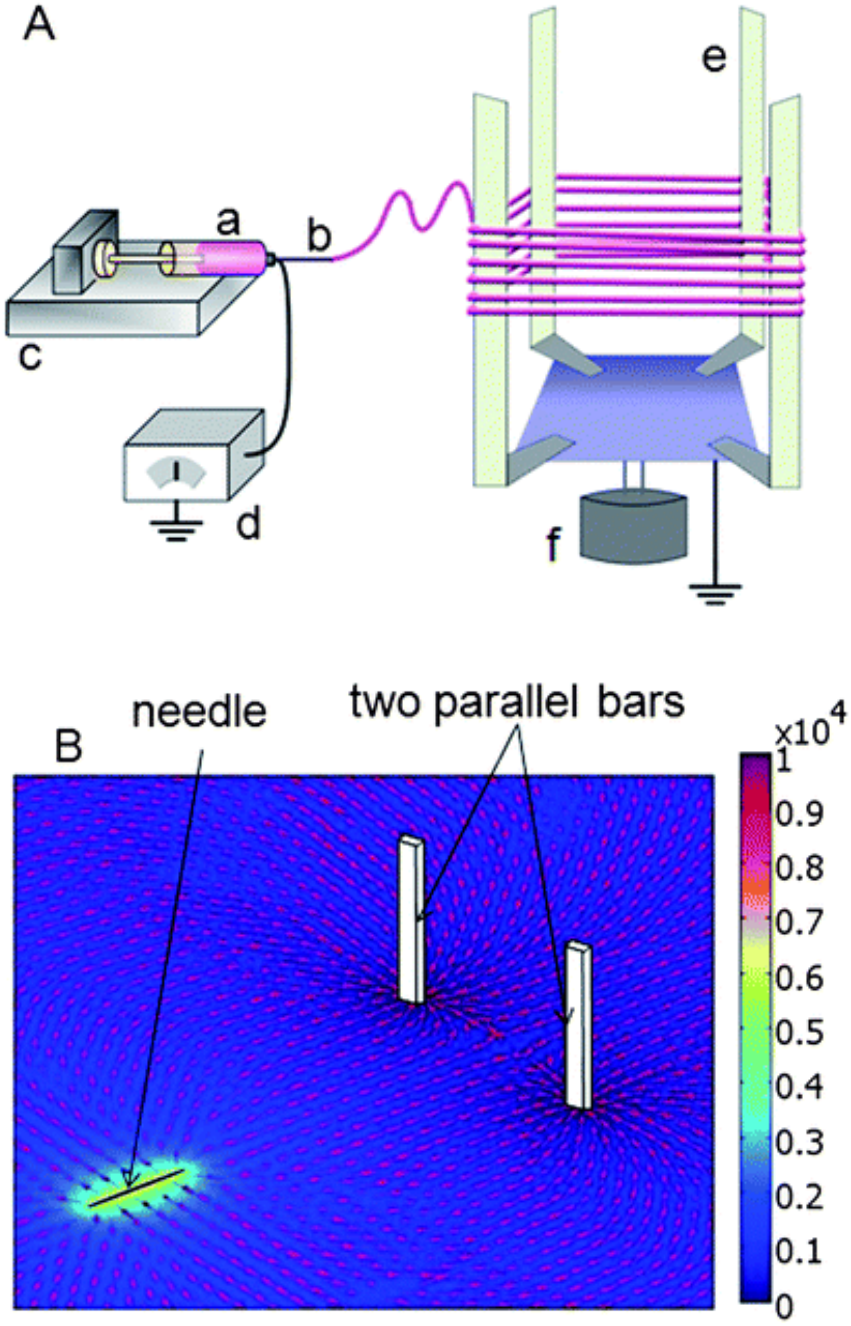


Figure 12. (A) Schematic diagram of the electrospinning apparatus for collecting nanofibers. (a) Syringe with polymer solution; (b) metal needle; (c) syringe pump; (d)

high-voltage power supply; (e) rotating collector; (f) motor. (B) The perspective view of electric field in the region between the needle and two-arm collector. The red arrows denote the direction of the electrostatic field, and different colors in the plane correspond to different potentials specified in the vertical bar. The needle is charged to 10 kV and the bars are grounded. (Reproduced from [122])

A positive voltage was applied to the needle until the Taylor cone emanating jets was produced. Upon collection of the microfibers from the mandrel, a home-made twisting device was used to fabricate the yarns.[122] The twisting device consists of two circular wire brushes with the ¾ inches diameter each, mounted co-axially on the holder.[122] Forty revolutions were applied to form the required yarn structure.

4.2.4. SEM characterization

The surface morphology of the CA/PMMA/PEO fibers and yarns were examined with a Hitachi Field Emission scanning electron microscope (FESEM-Hitachi 4800). The samples were sputter-coated with a few nanometer thickness of platinum prior to scanning electron microscopy (SEM) examination.

4.2.5. Yarn porosity

Yarn porosity was determined using the following methodology. A tributyl phosphate (TBP) droplet with the given volume was applied to the yarn. The droplet volume V_d was chosen greater than the yarn volume, $V_d > V_y$. The yarn volume was

calculated as $V_y = \pi R_y^2 L$, where R_y is the radius of yarn which was measured by the optical microscope Olympus BX-51, and L is the length of the yarn. The yarn was placed horizontally with one end attached in the TBP droplet. When the yarn was totally wet up to the other end, we took the yarn on a scale (Sartorius, BP-221S) for the mass measurement. TBP is used here because of its low vapor pressure (0.00012 mm Hg at 25°C) which prevents the liquid evaporation during measurements. TBP has density ρ = 0.979 g/cm^3. Porosity ε was calculated as $\varepsilon = (m_w - m_d)/(\rho V_y)$, where m_w is the mass of wet yarn, m_d is the mass of dry yarn, and V_y is the volume of yarn. The porosity of CA/PMMA/PEO yarn was examined on three samples and the average was calculated as ε=0.61±0.04.

4.2.6. Yarn permeability

The yarn permeability was examined by using the experimental setup shown in Figure 13a.[136] The yarn was attached to a highly permeable paper towel (Scott, Kimberly-Clark) at each end and was placed in a Π-shaped holder. The gap between two paper towels corresponding to the yarn length was l = 0.6 cm. Each end of paper towel was put in the liquid reservoir. The set-up works like a siphon: the hydraulic head (which was H = 3.8 cm) pushed the liquid to flow through the yarn from one container to the other. A scale (Sartorius, BP-221S) was used to identify the incremental change of the liquid mass in the container as a function of time. As shown in Figure 13b, the liquid flow stabilized after three hours. The mass loss was measured during four hours, and the resulting mass flux was: $Q = 2.38\times10^{-6}$ g/s. To find the permeability, k, we applied the

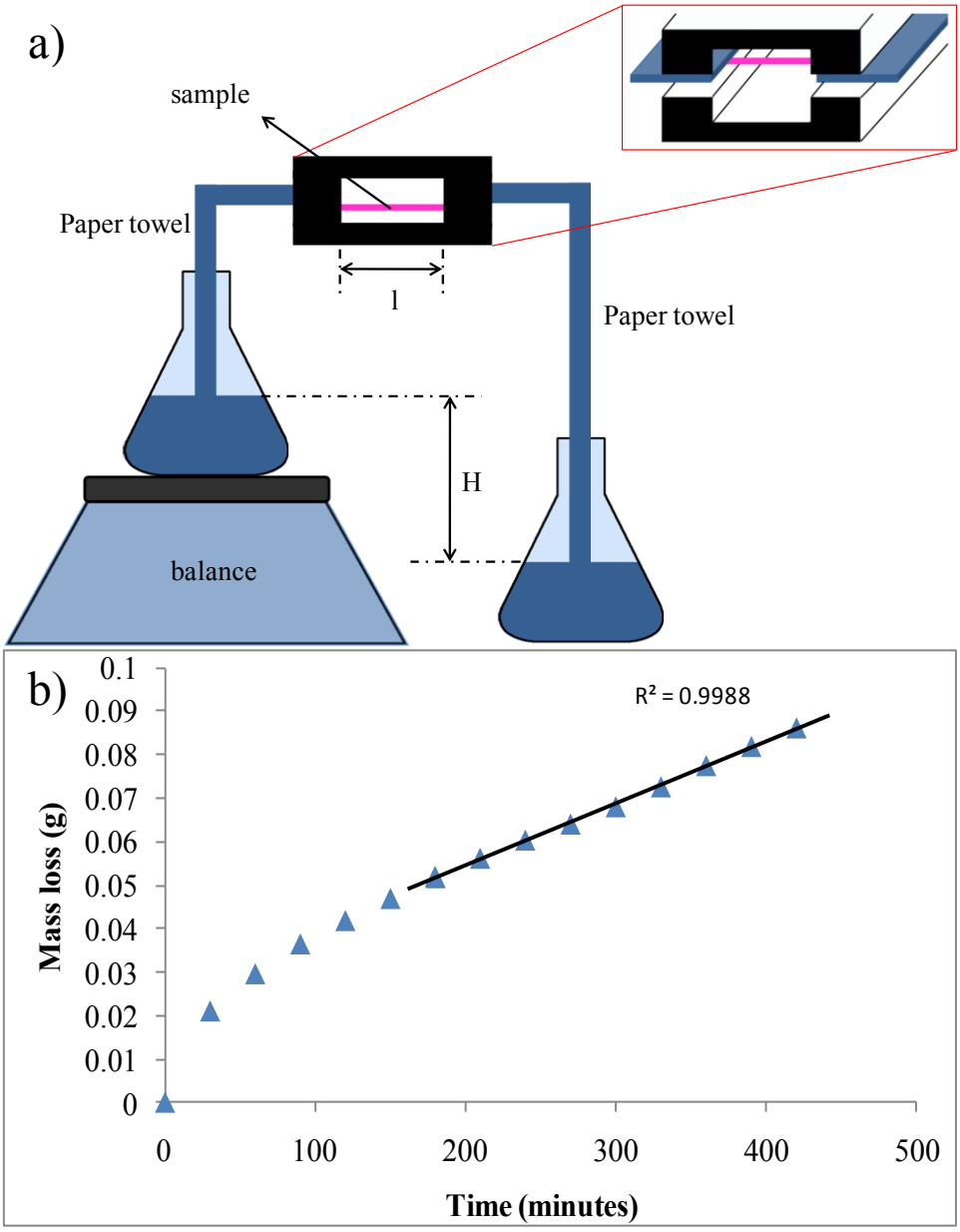


Figure 13. (a) The schematic set-up for permeability measurements. (b) The experimental mass loss as a function of time. It is clearly seen that the mass loss is almost linear after 3 hours stabilization.

Darcy law, $Q = \pi R_y^2(k/\eta)(\rho^2 gH)/l$, where R_y is the yarn radius, η and ρ are the viscosity and density of tested liquid, respectively, and g is the acceleration due to gravity. The properties of tested liquid (hexadecane) were obtained from material safety data sheets (MSDS) of the manufacturer (Acros Organics): density (ρ) = 0.773 g/cm^3, viscosity (η) = 3.03 cp. Substituting the experimental data, we obtain the permeability as $k = 8.84\times10^{-13}$ m^2. The scaling arguments suggest[137] that the interfiber distance in the yarn is measured in $d \sim k^{1/2} \sim 1$ μm.

4.2.7. Modification of the yarns

Fiber yarns were first cut into 2 cm fragments. Each of the pieces was then washed with 1 ml of RNAse®Zap reagent for 2 hours with vigorous shaking. The reagent contains surfactant and DNAse/RNAse inhibitors to remove any contamination possibly present on the surface. After treatment, the yarns were washed 2 times for 2 hours with 1 ml of DNAse, RNAse free water upon vigorous shaking to remove excess of RNAse®Zap from the fibers surface. Washed yarns were coated with streptavidin by either physical adsorption or covalent attachment. The amount of streptavidin used for coating was in 15-fold excess of the theoretical monolayer coating of the protein based on the effective surface of the yarns and dimensions of streptavidin molecule (4.5×4.5×5 nm).[138] Physical adsorption of streptavidin was done by incubating a 2 cm yarn piece with 50 μl of 1 mg/ml streptavidin solution overnight at room temperature. For covalent attachment, streptavidin was first activated by Sulfo-NHS-LC-Diazirine, a crosslinker that reacts with amino groups of a protein. 200 μL of 1 mg/ml solution of streptavidin

was incubated with 0.5 mg of the crosslinker for 1 hour in the dark at room temperature according to the manufacturer's instructions. After activation, excess of crosslinker was removed by washing streptavidin with TE buffer by centrifugation two times through the 10,000 molecular weight cut off (MWCO) centrifugal devices. Yarns were then incubated with activated streptavidin for 20 minutes in the dark at room temperature followed by ultraviolet (UV) irradiation for 20 minutes (UVGL, 365 nm, 16 Watt lamp, Ultra-Violet Products Ltd, Cambridge, UK). Under the UV irradiation, diazirine groups of activated streptavidin form reactive carbene intermediates capable of reacting with C-H bonds on the polymer fiber surface. After UV treatment, yarns were stored in the dark at room temperature for 30 minutes to allow the reaction to complete. After modification with streptavidin, yarn pieces were washed with 1 ml of TE buffer for 1 hour × 2 times to remove any unbound protein.

To determine binding yield of streptavidin, it was labeled with AlexaFluor®594 dye. For this procedure, 0.1 mg of dye was mixed with 200 µl of 1 mg/ml streptavidin solution in TE buffer and incubated for 1 hour covered with foil at 37°C. Then labeled streptavidin was purified two times with TE buffer using 10,000 MWCO centrifugal devices. After labeling, streptavidin was incubated with yarn piece for physical adsorption directly or activated with the crosslinker as it was described above for covalent attachment. The binding yield was determined from fluorescence of streptavidin solution before and after protein adsorption.

Fluorescent imaging was also performed to compare uniformity of streptavidin coating on the yarns for covalent and physical adsorption. For this experiment, fibers coated by fluorescently labeled streptavidin were washed in 1 ml of TE buffer 2 times for 1 hour and in TE buffer with 0.3M NaCl and 0.1% SDS 2 times for 30 minutes and imaged using fluorescent microscope.

At the final step of modification, 20 µl of 1 mM solution of biotin-oligo dT25 complex was used to introduce mRNA capturing ligands on the surface of the yarns. Molar ratio of biotin-oligo dT25 to initial amount of streptavidin was 8:1. To remove any unbound biotin-oligo dT25 molecules, the yarns were washed 2 times with 1 ml of DNAse, RNAse free water for 30 minutes. Modified fiber yarns were used for mRNA extraction within few hours after their preparation.

4.2.8. Binding capacity of oligo dT_{25} modified fiber yarns

To determine binding capacity of the oligo dT_{25} modified fiber yarns we studied attachment of synthetic fluorescently labeled oligo dA_{25} nucleotide to the modified fiber yarns. The yarns were cut into 0.5 cm pieces and weighed prior to experiments. Fiber yarn pieces were then incubated with 20 µl of fluorescently-labeled poly A_{25} at different concentrations (30 min at room temperature). The following concentrations of nucleotide were used in this experiment: 0, 1, 10, 100, 250, 500, 750 and 100 mM. After the incubation, fibers were carefully removed and remaining solution was centrifuged for 30 s at 1000 rpm to precipitate any loose fiber fragments. 15 µl of the solution was then collected from the centrifuge tube and it fluorescence was compared to that of the same

amount of the fluorescence obtained from corresponding initial solution. Total amount of nucleotide, n_i, bound to the fiber yarn (nmol) for each concentration, i, was calculated using the following formula:

$$n_i = \frac{F_1 - F_2}{F_1} * N_i ,$$

where F_1 and F_2 are fluorescence intensities of polyA_{25}-fluorescein solutions before and after the incubation correspondingly, N_i – total amount of nucleotide present in the corresponding solution, nmol. Total of 3 replicates were made for each concentration of nucleotide solution.

4.3. RESULTS AND DISCUSSION

4.3.1. Characterization of CA/PMMA/PEO yarns: specific surface area, porosity, and permeability

Figure 14a shows the structure of typical CA/PMMA/PEO yarn after applying 40 twists. Figure 14b shows the surface morphology of CA/PMMA/PEO fibers. The yarn diameters were analyzed using the ImageJ software (NIH) and showed 5±0.5 μm diameter averaged over twenty tested microfibers in the picture.

The specific surface area was defined as the surface area (m^2) per unit weight (g). Assuming that the yarn consists of the fibers with the same average diameter, one can estimate the specific surface area (S):

$$S = \frac{A}{W} = \frac{\pi DL}{1/4\pi D^2 L\rho} = \frac{4}{\rho D},$$

where A is the fiber surface area; W is the fiber mass; D is the fiber diameter; L is the fiber length. The density was calculated assuming that the solvent was totally evaporated and only solid polymers were present in the fiber. Hence, the overall density:

$$\rho = \frac{\sum m_i}{\sum \frac{m_i}{\rho_i}} = 1.245 \text{ g/cm}^3,$$

where m_i is the mass of each component in the fiber and ρ_i is the density of each component in the fiber. The densities of CA, PMMA, and PEO are 1.3 g/cm^3, 1.2 g/cm^3, and 1.21 g/cm^3 from the manufacturer MSDS, respectively. The mass fraction of each component is described in section "Dope preparation".

Hence, the specific surface area (S) was estimated as:

$$S = \frac{4}{\rho D} = \frac{4}{1.245*10^6 \times 5*10^{-6}} = 0.64 \text{ m}^2/\text{g}.$$

Porosity of CA/PMMA/PEO yarn was examined on three samples as discussed in Sect. 4.2.5. The average porosity (ε) of these yarns was found to be 0.61±0.04. The permeability of the produced yarn was analyzed as discussed in Sect. 4.2.6 and was estimated to be $k = 8.84\times10^{-13}$ m^2. The scaling arguments suggest[137] that the interfiber distance ($d \sim k^{1/2}$) in the yarn is about 1 μm.

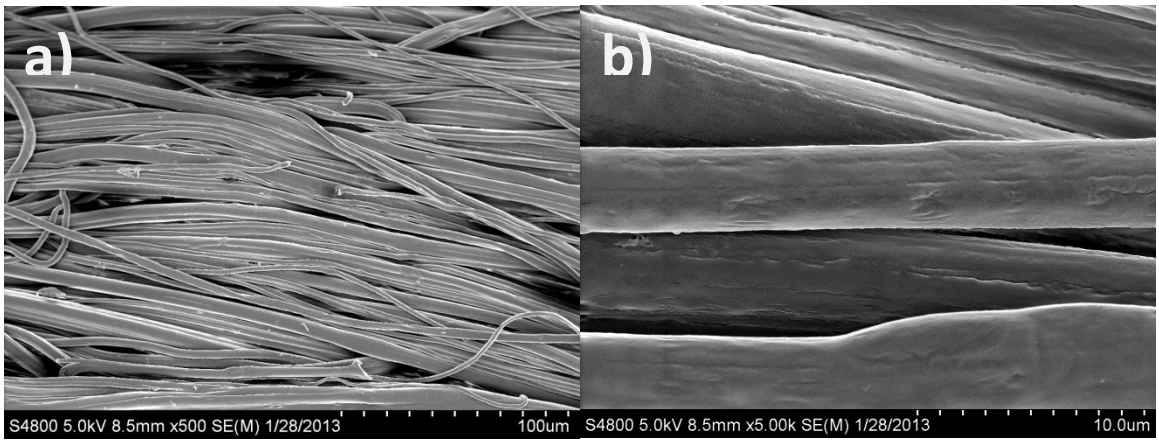


Figure 14. SEM images of typical CA/PMMA/PEO yarns at different magnifications.

4.3.2. Modification and determination of binding capacity of oligo dT_{25} modified fiber yarns

We have tested several materials to manufacture fibers for extraction of mRNA from cell lysates. These materials included nylon (commercially available), and electrospun CA/PMMA/PEO, CA/PMMA, CA/PAN and PAN/PMMA prepared as described in the Experimental section. Prepared yarns were different by several parameters: material of the fibers, yarn thickness, fiber thickness and a number of twists. Experiments with fluorescently labeled streptavidin demonstrated that streptavidin can be successfully immobilized on the surface of all fibers by either adsorption or covalent binding. Comparison for physical adsorption and covalent attachment of streptavidin on the surface of CA/PMMA/PEO fibers is shown on the Figure 15.

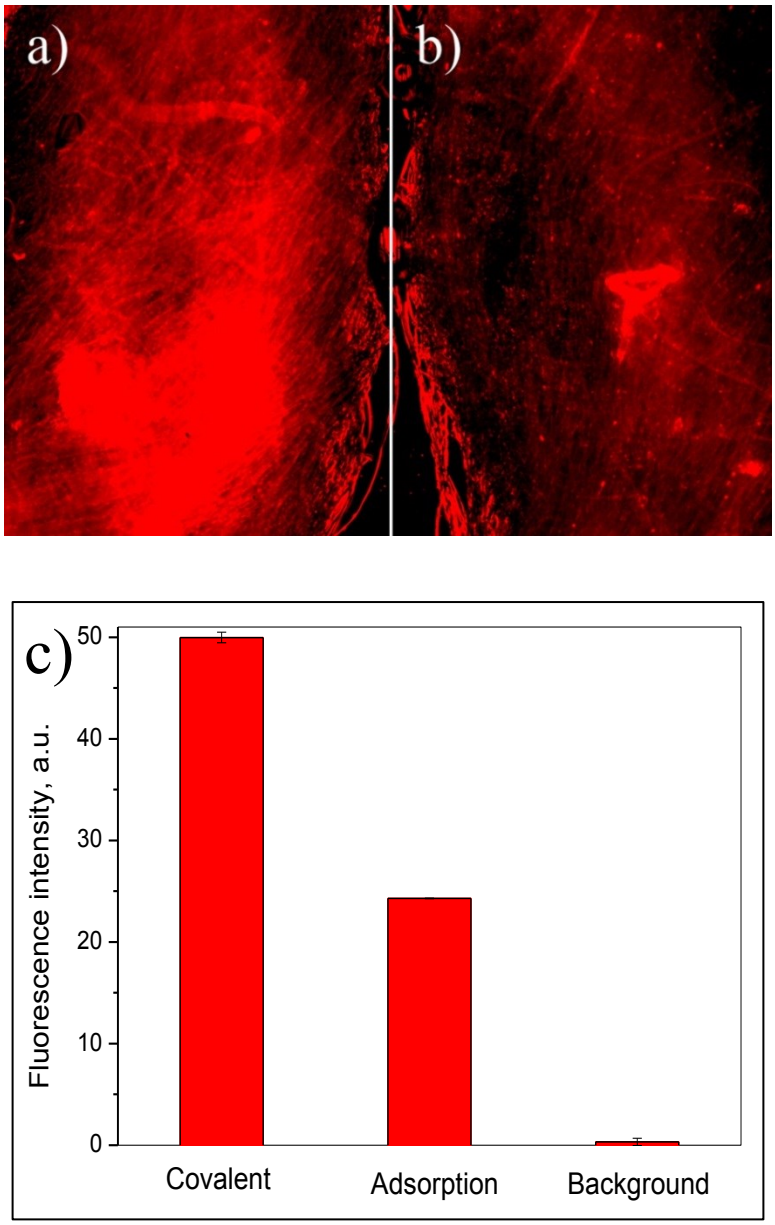


Figure 15. Fluorescent images of CA/PMMA/PEO fiber yarns coated with AlexaFluor®594 labeled streptavidin using (a) covalent attachment and (b) physical adsorption. (c) Relative average intensity of fluorescence: covalent to physical adsorption and to the background. Images were taken after washing poorly attached streptavidin.

Image was taken after washing steps as described in the Experimental section, and the image indicates that the fluorescence signal from the fiber with covalently attached streptavidin is higher and more uniform than that from the fiber with physically adsorbed streptavidin. Quantitative analysis of the images using ImageJ software showed that fluorescence intensity in the case of covalently attached streptavidin was ~2.1 times higher than that for physically adsorbed protein. The streptavidin coating remained on fibers surface even after washing with TE buffer that contains 0.1% SDS surfactant, indicating strong immobilization of protein on the fibers surface. Total binding efficiency of covalently attached streptavidin was calculated based on the fluorescence drop of initial solution after incubation and consisted of 37±7 μg per mg of fiber yarn.

Figure 16 shows binding of $polyA_{25}$ nucleotide to the surface of oligo-dT_{25} modified fiber yarns as function of concentration. Assuming that saturation in Figure 16 occurs when all oligo dT_{25} fragments are bound to $polyA_{25}$, binding capacity for $polyA_{25}$ was calculated as 80±9 nmol per mg of fiber (n=3). It is expected that mRNA binding capacity of the yarns is similar; however binding mRNA may take longer time to achieve because mRNA molecules are larger than $polyA_{25}$ and their attachment to the fiber surface may be diffusion limited.

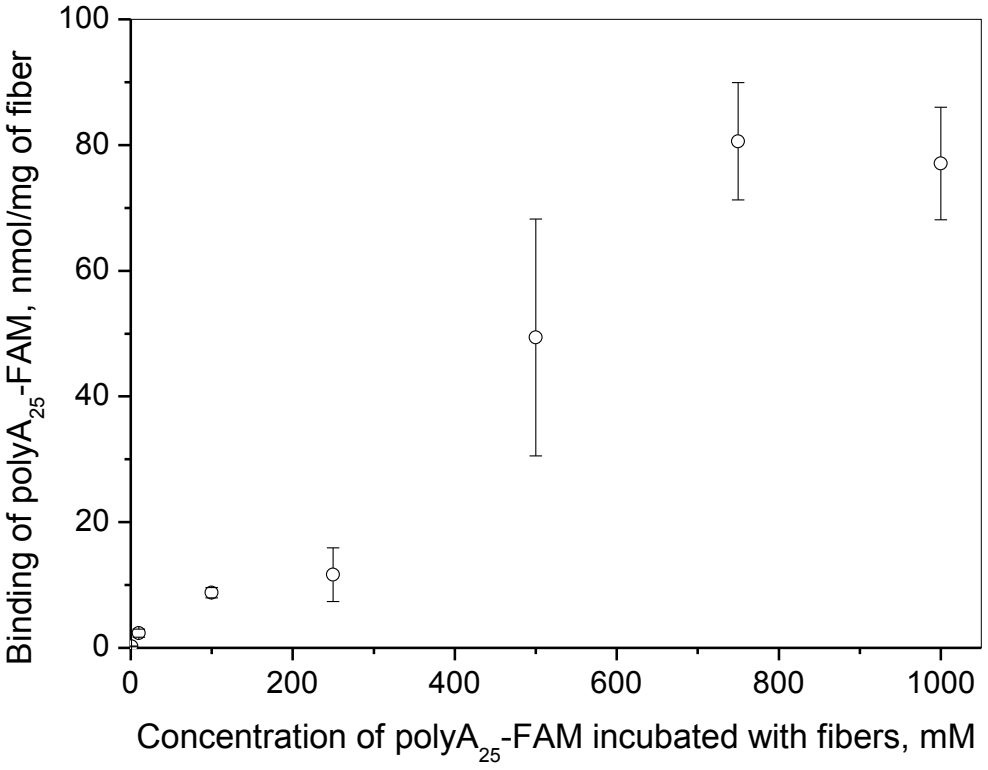


Figure 16. Binding of $polyA_{25}$ to 0.5 cm fiber yarn pieces. Saturation is achieved at 79±8 nmol per mg fiber (n=3).

Overall, prepared fiber-based devices possess necessary amount of binding sites and desired microfluidic properties for analysis of the single cells. Testing of the prepared fiber yarns biosensors for mRNA extraction from cell lysates and possibly single cells will be performed in the Chapter 5.

4.4. CONCLUSIONS

In conclusion, we have successfully performed preparation of CA/PMMA/PEO fiber yarns by electrospinning method. Fiber yarn bundles were characterized with various approaches and yielded following physical properties: porosity = 0.61, permeability coefficient $k=8.84\times10^{-13}$ m^2, estimated specific surface area = 0.64 m^2/g. We have found that the covalent attachment of streptavidin on the surface of fiber-based devices is more suitable for our applications and extensive washing will not remove streptavidin molecules from the surface of yarns. These fibers were prepared to serve as mRNA extraction devices and therefore we measured the loading efficiency of the fiber yarns using fluorescently labeled polyA_{25} molecules. We have found that the limiting capacity of the mRNA loading on the fiber surface is approximately 80 nmol per mg of fiber, which is excessive compare to the total mRNA from a single cell. Therefore oligo dT_{25} modified fiber-based devices can be used for the future testing in the mRNA extraction experiments.

CHAPTER 5

EXTRACTION OF MRNA FROM CELL LYSATES USING OLIGO DT_{25} MODIFIED FIBER-BASED DEVICES

5.1. INTRODUCTION

Profiling of messenger ribonucleic acid (mRNA) expression remains the most common method for evaluation of gene expression.[119] Most techniques are based on amplification of mRNA using polymerase chain reaction (PCR).[78] PCR is broadly used in a variety of applications, including diagnosis of genetic disorders, forensic science, and detection of antibiotic-resistant pathogens to name a few.[139] While PCR is a well-developed and widely used technique, its implementation is time consuming and requires specially trained and experienced personnel. Much effort has therefore been directed towards development of faster and simpler variations of PCR, with the "bedside PCR" in clinician's office being the ultimate goal that is yet to be achieved. Development of miniature microfluidic PCR devices has become one of the main focus areas of this research because their smaller size and specially designed architecture allow faster PCR analysis.[121] However, PCR-on-a-chip microfluidics is rather expensive and their use requires even more rigorous training/expertise than in the case of conventional PCR. Further on, an integration of the microfluidic PCR with sample preparation and detection of the products still remains a challenge.[121]

Recently, fiber-based microfluidic devices were proposed as alternative to on-chip microfluidic devices.[122] In fiber-based devices, liquid flow is controlled by capillary forces in micro- or nanofiber yarns.[122, 123] Besides improved manipulation of the liquid flow, fiber-based microfluidic devices are easy to fabricate and can be surface modified to perform a variety of functions. From the standpoint of PCR analysis, one apparent advantage of fiber-based devices is the possibility to collect the genetic material and process it on the same platform. Other potential advantages include ease of use, low cost, time saving, and possibility of focused collection of genetic material from a small area of interest. Since diameter of the fiber yarns can be as small as few microns, it is possible, in principle, to use such fiber-based devices for collecting mRNA from extremely small areas of interest, ultimately from individual cells. Thus, the goal of this work was to manufacture the fiber-based PCR sampling devices and evaluate their performance using conventional PCR procedure as the "gold standard". We hypothesized that because of their high specific surface area and ability to specifically bind to mRNA, the fiber-based devices would prevent loss of genetic material inevitable during the mRNA isolation in conventional PCR procedure thus leading to high sensitivity. To verify this hypothesis, we produced the microfiber yarns for the PCR analysis of highly diluted cell lysates with mRNA concentrations corresponding to those found in individual cells.

Schematic of the fiber-based mRNA extraction devices performance is shown in Figure 17.Initially fiber yarns were modified with streptavidin and then biotin oligo dT_{25}.

Primary vascular smooth muscle cells were isolated from adult rats and maintained in the cell culture up to passage number 8-10. After that the starvation of them performed for the course of two weeks and then lysates were prepared from both cell cultures. Prepared lysates were analyzed by conventional method as well as by using ologo dT_{25} modified fiber yarn devices. As a model system for gene expression analysis, we used vascular smooth muscle cells (VSMC). These cells have two different phenotypes – synthetic and contractile. The cells with synthetic phenotype are characterized by spread morphology and have high proliferation rate. Contractile cells have a spindle-like shape and do not proliferate. This model is clinically important because transition from the contractile to synthetic phenotype is associated with restenosis – the overgrowth of tissue in a vascular stent that blocks the blood flow. The two phenotypes show different levels of expression for a number of genes. For example, contractile (healthy) cells have a higher level of α-smooth muscle (SM) actin expression, while synthetic (pathological) cells have a decreased level of α-SM actin expression.[140] The contractile phenotype has higher expression levels of other structural proteins such as γ-actin, β-actin, and smooth muscle myosin heavy chain compared to the synthetic phenotype.[140] Based on this difference, we used this cell model to show that mRNA extraction using the fiber-based device can be performed for quantitative analysis of gene expression at concentrations of genetic material corresponding to a single cell.

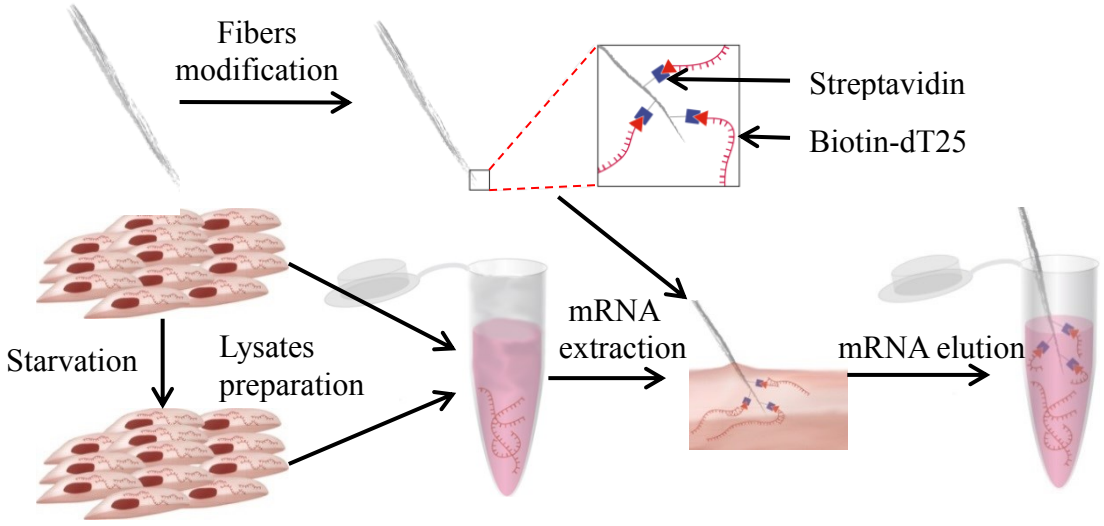


Figure 17.Schematic of mRNA isolation using oligo dT_{25} modified fiber-based device.

5.2. Materials and Methods

5.2.1. Materials

Cellulose acetate (CA, MW: ~37 kDa), polyacrylonitrile (PAN), poly(methyl methacrylate) (PMMA, MW: ~120 kDa) and polyethylene oxide (PEO, M_w=1,000 kDa) were purchased from Sigma-Aldrich Corporation. Dimethylacetamide (DMAc) was purchased from Alfa Aesar. Nylon yarn with thickness about 0.3 mm was purchased from Middleburg Yarn Company. The yarn (containing ~170 filaments with diameter of ~25 μm), had estimated surface area of 1385 cm^2/g. Streptavidin was purchased from Rockland Immunochemicals, Inc. Biotin-oligo dT_{25}, and primers for targeting and housekeeping genes were purchased from Integrated DNA Technologies, Inc. DNAse,

RNAse and Protease free water, Tris EDTA (TE) buffer, sodium chloride and sodium dodecyl sulfate (SDS) were purchased from Acros Organics. RNaseZap®, TURBO DNA-free™ Kit and RETROscript® Kit were purchased from Ambion, Inc. RNeasy Mini Kit and QuantiTect SYBR Green PCR Kit were purchased from QIAGEN, Inc. Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS) and Antibiotic/Antimycotic solution were purchased from Cellgro, Mediatech, Inc. Poly A25-Fluorescein was purchased from Integrated DNA Technologies, Inc. All other chemicals were purchased from Sigma-Aldrich and used as received unless otherwise specified.

5.2.2. Cell culture models

Primary cell culture line of rat aortic smooth muscle cells was collected from female rats as described below. Adult female Sprague-Dawley rat was euthanized using 100% CO_2 flow for 15 minutes following with bilateral pneumothorax to ensure death. Aorta was carefully washed with Hank's Balanced Salt Solution several times to remove any excess of blood and excised from the rat. After that it was carefully scrapped with a scalpel under microscope to remove tunica intima and tunica adventitia, leaving tunica media which contains vascular smooth muscle cells in the collagen matrix. The collagen matrix was then dissolved using collagenase solution and cells were allowed to migrate from aorta to the Petri dish overnight. After this step, migrated cells were removed from the surface of the Petri dish with 0.25% trypsin solution and passaged in T-75 flasks. Cell culture was maintained using DMEM with 10% FBS and 1% antibiotic/antimycotic

solution. Cells were grown till 85-90% confluency before passaging and cell culture was grown till the passage number 8-10. After reaching high passage number the change in phenotype of the cells was induced by starvation of cells from FBS.

15-day starvation period was chosen to ensure cells underwent the phenotypical change from synthetic to contractile phenotype.[140] After starvation, cell shape changed from round to mostly triangular or spindle-like and these cells were used as a model of contractile phenotype. FBS-fed cells were used to obtain synthetic phenotype. Starved and fed cells were collected by trypsinization and washed with sterile PBS buffer several times. Cell numbers were determined using a cell counter (Z™ Series COULTER COUNTER®, Beckman-Coulter, Inc., Brea, CA, USA) with detection range set for of 10-40 microns. Cell lysates were prepared using an ultrasound homogenizer (Omni Ruptor 4000, Omni International, Kennesaw, GA, USA), aliquoted and stored in -80°C freezer.

Primary cell culture lines of adult and neonatal rat myocytes were gifted by Dr. Bruce Gao, (Dept. Bioengineering, Clemson University). The cells were washed with sterile PBS several times to remove remaining medium, resuspended in sterile PBS buffer and counted using the cell counter. Cell cultures were then lysed using the ultrasonic homogenizer, aliquoted and stored in -80°C freezer.

5.2.3. Processing of cell lysates

Extraction of total RNA from cell lysates was performed using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol. After extraction of total RNA it was treated with TURBO DNA-free™ Kit to remove any DNA contamination. Amount of total RNA was measured using absorbance of its solution at 260 nm and consisted of 290±30 μg per 10^7 cells. Ratio of absorbance 260/280 for total RNA solution was measured and found to be in the range of 1.98-2 indication pure RNA solution. Reverse transcription reaction was performed using RETROscript® Kit (Life Technologies, Grand Island, NY) to prepare cDNA for further use in PCR amplification reactions.

5.2.4. mRNA extraction using fiber-based probes

Extraction of mRNA molecules that have a poly-A tail was performed using the following protocol. 2 cm yarn fragments with immobilized oligo dT_{25} were incubated with 50 μL of cell lysates at different dilutions (corresponding to 10^7 – 1 cell) for 30 minutes at room temperature in 10 mM Tris 1 mM EDTA (TE) buffer with 0.3 M NaCl and 0.1% SDS. After the incubation step, fibers were removed from lysates and washed with 1 ml of TE buffer with 0.3 M NaCl and 0.1% SDS total of 2 times for 30 minutes.

The buffer was chosen based on the literature data[141]: high salt concentration prevents decomposition of poly-A – poly-T bonds; while surfactant removes any non-specifically adsorbed RNA. After washing, elution of mRNA was performed using 10 μl

of plain TE buffer with excess of oligo dT_{25} (2 µl of 1 mM solution) at 70°C for 10-15 minutes. Eluted mRNA was then used in reverse transcription reaction using RETROscript® Kit. Reverse transcription was carried out for 1 hour at 44°C followed by inactivation of the enzyme at 95°C for 10 minutes. Fiber-based mRNA sampling devices are used for both sample collection and to replace the column as mRNA carriers in the washing step. Use of the fiber-based devices also eliminates DNAse treatment step. Overall, the use of fiber-based devices for mRNA extraction is easier to perform and faster than conventional PCR; it requires less equipment (no columns) and reagents (Figure 18).

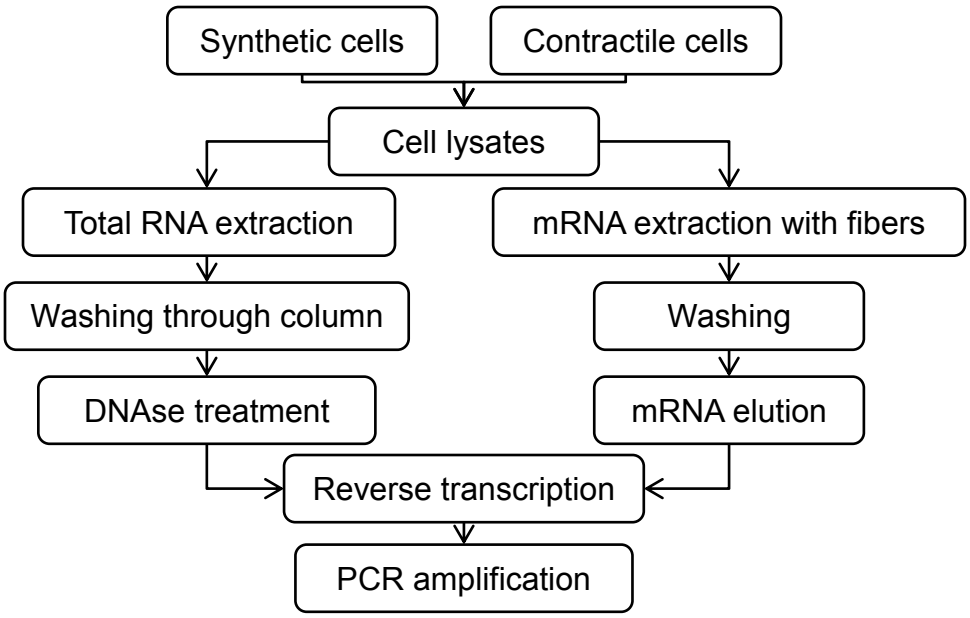


Figure 18**.** Schematic of the steps in conventional PCR procedure (left) and using fiber-based mRNA extraction devices (right).

5.2.5. mRNA extraction from tissue samples

A pilot experiment for extraction of mRNA from tissue samples was performed using the following procedure. First, the heart and aorta from adult Sprague-Dawley rat were excised and washed in sterile PBS solution. Abdominal aorta was cut along the vertical axis to open it up and remove the endothelial cells layer with careful scraping of the inner surface with the scalpel blade.

Modified fiber yarn piece, total of 2 cm in length and soaked in binding buffer was inserted in to the lumen of the artery with exposed vascular smooth muscle cells and incubated for 30 minutes. Another yarn segment was incubated within 3-5 mm deep incision that was made in the left ventricular wall of the heart. The last sample was incubated with minced tissue of left ventricular wall of the heart. After incubation with tissue samples, fibers were removed and thoroughly washed with binding buffer as it was described above. mRNA elution and RT following by PCR reaction was carried out.

5.2.6. mRNA extraction procedure using magnetic beads

Magnetic beads supplied by Quanta biosciences were used as a control method for mRNA extraction and analysis. First, total RNA was extracted from cell lysates samples using conventional method according to the manufacturer's manual. Extracted total RNA was then incubated with magnetic beads in mRNA extraction buffer for 10 minutes at room temperature and 3 minutes at 80°C. After mRNA was attached to the surface of beads, they were spun down at 8000 ×g for 1 minute and washed 2 times with washing

buffer provided in the kit. Following by careful removal of all the liquid from the beads pellet, samples were dried in the air flow for 5-10 minutes. Elution of mRNA was performed using the necessary amount of eluting buffer, following with magnetic beads removal by centrifugation. Supernatant, containing extracted mRNA was first used in RT reaction and products were analyzed by PCR.

5.2.7. PCR procedure and data analysis

PCR analysis was performed using QuantiTect SYBR Green PCR Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's manual in Rotor-Gene 3000 Thermal cycler (Corbet Research, part of Quiagen Inc., Valencia, CA). PCR amplification was performed using the following thermal cycling: 94°C for 20 sec, 60°C for 20 sec and 72°C for 15 sec, with number of cycles of 50. The threshold PCR data was analyzed by the method of relative quantification of gene expression proposed in Ref. [142]. The threshold level was set sufficiently above the background and the number of cycles required to reach the threshold was registered. C_T value for each sample fluorescence curve was chosen at the intersection of the fluorescence curve with the threshold line. Sample graph for the fluorescence versus C_T number is shown in Figure 19.

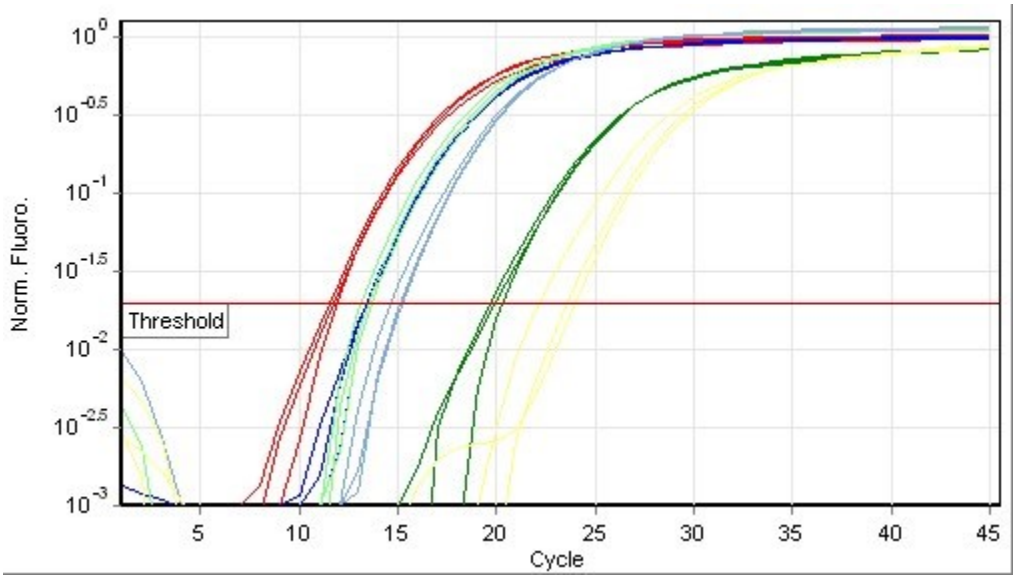


Figure 19. Sample graph displaying normalized fluorescence dependence from cycle number in PCR analysis. Red line represents threshold level chosen (0.02).

Number of copies (N) that were produced in PCR reaction will be estimated as following: after each cycle, there will be doubling of the PCR product, leading to the assumption that after *n* cycles there will be:

$$N_f = N_0 \times (1+Y)^{n-1},$$

where N_0, N_f – initial and final number of copies of the investigated gene, Y – the efficiency of PCR reaction. [143] Average C_T value represents the amount of mRNA that was present in the sample, which is proportional to Y^{CT}.

First, we calculated expression of the targeted gene which was related to the expression of the housekeeping gene in fed cell culture - $\Delta C_{T\ fed}$ = (avg. $C_{T\ fed,\ A}$ - avg. $C_{T\ fed,\ B}$). Same ΔC_T was calculated for the starved cell culture. To determine, in which cell culture there was an increased expression of the chosen genes, we have calculated the difference between these two values ($\Delta\Delta C_T$). The relative gene expression for the vascular smooth muscle cells was calculated using the following formula:

$N_{starved}/N_{fed} = Y^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ fed} - \Delta C_{T\ starved}$ = (avg. $C_{T\ fed,\ A}$ - avg. $C_{T\ fed,\ B}$) – (avg. $C_{T\ starved,\ A}$ - avg. $C_{T\ starved,\ B}$),

where $N_{starved}$, N_{fed} are the initial numbers of β-actin gene copies in starved and fed cells correspondingly, and avg. $C_{T\ fed,\ A}$, avg. $C_{T\ starved,\ A}$, avg. $C_{T\ fed,\ B,}$ avg. $C_{T\ starved,\ B}$ are the averaged C_T values that were calculated from 3 PCR replicates for targeted gene A or housekeeping gene B from fed or starved VSMC culture, respectively. Same formula was used for determination of gene expression ratio in adult and neonatal myocytes cell cultures.

The PCR efficiency for targeting and housekeeping genes primers was calculated from the C_T values of diluted genes with known concentrations by using the following expression, which was introduced by Livak K. [142]:

$Y = 10^{-1/k}$.

PCR efficiency is the characteristic of PCR reaction, ideally, 100% efficiency meaning that in each PCR cycle there was a doubling of the amount of the amplified

sequence. This approach allows one to use a variable amount of cells for experiments as we use the housekeeping gene to monitor relative gene expression in different types of cells.

5.2.8. Statistical analysis

The values of genes expression ratios for different samples were compared using the paired t-test (P=0.05). The number of replicates for the mRNA extraction experiments using modified fiber yarns or total RNA from cell lysates was n=3.

5.3. RESULTS AND DISCUSSION

5.3.1. Gene analysis

We identified several genes that could serve as potential targeting (α-smooth muscle actin (α-SM actin), β-actin, smooth muscle myosin heavy chain (SM MHC)) and housekeeping (β-2-microglobulin (β-2-MG), L32, hypoxanthine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-Tubulin, Tubulin T) genes in our experiments. List of the primers for chosen genes is shown in the Table 1. To pick up the appropriate genes for this proof-of-concept study, we performed an experiment to establish efficiency of the primers for the above genes. Experiment was done by dilution of cDNA template made from total RNA isolated from the cell cultures for all of the corresponding primers following with PCR analysis. The resulting cycles to threshold (C_T) values were plotted as a function of Log (concentration of loaded cDNA template) (Figure A 1-9). These dependencies were found to be non-linear for the

following genes: α-Tubulin, Tubulin T, HPRT, GAPDH, and smooth muscle myosin heavy chain (SM MHC). Such a non-linear relationship in samples with different concentration of cDNA template is associated with large error in experimentally determined gene concentrations. Therefore, these genes were excluded from the list of the candidates.

Also, by running gel electrophoresis on the PCR products, we have found that α-Tubulin primers have been amplifying multiple DNA segments with different number of base pairs (Figure 20). Graphs for the efficiency of primers could be accessed in Appendices section. After exclusion of these genes, we were left with two possible target genes, α-SM actin and β-actin, and two possible housekeeping genes, β-2-MG and L32.

As it was shown in the table 1, the efficiency for both chosen genes was found to be very close to 100%, meaning that we can use the double delta CT method that was described first by Pfaffl, M.V. [144] This method was taking in assumption that the PCR efficiency is very close to a 100% and therefore the formula for relative gene expression:

$N_{starved}/N_{fed} = E^{-\Delta\Delta CT}$, could be written as: $N_{starved}/N_{fed} = 2^{-\Delta\Delta CT}$,

which simplifies calculations to compare the expression levels for both targeting and housekeeping genes expressed in both cell cultures.

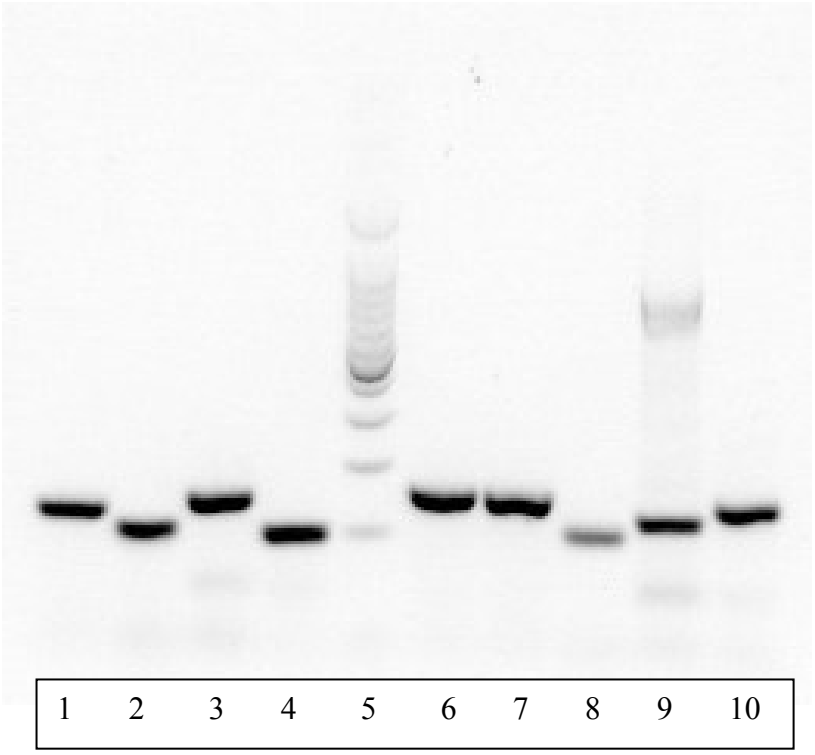


Figure 20. Gel electrophoresis of PCR products for different primers. Line 5 is 100 bp DNA ladder (lower band corresponds to 100 bp, then 200 bp , intensive band in the middle correspond to 500 pb, and the last band corresponds to 1500 bp). Genes: 1 – α-SM-actin, 2 – β-actin, 3 – β-2-microglobulin, 4 – L32, 5 – HPRT, 6 – GAPDH, 7 – SM MHC, 8 – α-Tubulin, 9 – Tubulin T.

Table 1**.** List of primers that were used in the present work.

#	Gene name	Primer sequence	Gene type	Efficiency
1	α-SM-actin	Forward GAGACCCTCTTCCAGCCATC Reverse CCCGAGAGGACGTTGTTAGC	Targeting	77.8%
2	β-actin	Forward TGATGGTGGGTATGGGTCAG Reverse GACAATGCCGTGTTCAATGG	Targeting	98.4%
3	β 2 micro-globulin	Forward GGTGACCGTGATCTTTCTGG Reverse GTGGGTGGAACTGAGACACG	House-keeping	78.3%
4	L32	Forward AGTTCATCAGGCACCAGTCG Reverse CTGGCCCTTGAATCTTCTCC	House-keeping	100%
5	HPRT	Forward AAGAGTCCTGTTGATGTGGCCAGT Reverse CTGCCTACAGGCTCATAGTGCAAA	House-keeping	92.3 %
6	GAPDH	Forward AAGAGTCCTGTTGATGTGGCCAGT Reverse CTGCCTACAGGCTCATAGTGCAAA	House-keeping	92.3 %
7	SM MHC	Forward TTGGCAAGTTCATTCGCATC Reverse CCCTAGCCTGTCGAATAGCC	Targeting	80.6 %
8	α-Tubulin	Forward CTGTCATGGTGGACCTGGAG Reverse GCGGGCGTAATTGTTAGCTG	House-keeping	112 %
9	Tubulin T	Forward AGACCCAAGGCAGCAAGAAG Reverse TGGTAACTTCCAGTGGGATCG	House-keeping	94.7 %

However, rat α-SM actin and β-2-MG mRNA molecules have significantly shorter poly-A tails than β-actin and L32 mRNA molecules, according to the gene database.[131] The shorter poly A tail length will lead to lower affinity of mRNA molecules to the oligo dT_{25} coated probes and possibly different extraction rates. Also the PCR efficiency for the chosen set of primers of α-SM actin and β-2-MG was not close to 100%, which will introduce additional calculation steps and probably increase of chance of calculation errors. Thus we decided to use β-actin as the targeting gene and L32 as a housekeeping gene. The ratio for expression of targeted β-actin gene in starved and fed VSMC was calculated using the following formula[142]:

$N_{starved}/N_{fed} = 2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T =$

$(\text{avg. } C_{T\ \text{fed, }\beta\text{-actin}} - \text{avg. } C_{T\ \text{fed, L32}}) - (\text{avg. } C_{T\ \text{starved, }\beta\text{-actin}} - \text{avg. } C_{T\ \text{starved, L32}})$.

The same formula was used to determine gene expression ratio for adult and neonatal rat myocytes. This formula provides relative expression of targeted gene in different cell types and there is no need to analyze exactly the same amounts of cells, because gene expression of target gene is related to the expression of housekeeping gene. The ratio of expression levels of targeting and housekeeping gene for both starved and fed VSMC, and neonatal and adult myocytes obtained from cell lysates was found to be 4.30±0.06 and 0.95±0.04, respectively.

Higher β-actin expression in starved VSMC compared to fed culture is in good agreement with the literature[145] due to the fact that starved cells are stiffer and express

more cytoskeletal proteins. For myocytes, relative gene expression in neonatal and adult cells was not found to be significantly different. Indeed, β-actin gene has been used as a housekeeping gene in these cell cultures according to the literature data.[146]

Efficiency of PCR for targeting β-actin gene was found to be 98.4%, and for housekeeping gene L32 ribosomal protein – 100%.Although all yarns demonstrated some ability to extract mRNA from cell lysates, quantitative ratios for gene expression by serum-starved and serum-fed VSMCs differed considerably for the fibers manufactured from different materials. The protocol for extraction of mRNA from cell lysates was the same for all fibers and is discussed in the experimental section. Figure 21 shows relative expression of β-actin mRNA by serum-starved and serum-fed VSMCs for different fiber materials. After initial testing with the cell lysates it was found that the fibers coated by covalently attached streptavidin showed rather consistent C_T values, whereas fibers modified by physical adsorption were showing larger error and poor batch-to-batch reproducibility (data not shown). Thus only fibers with covalently immobilized streptavidin were used for all further experiments. Notably, presence of oligo-dT mRNA capturing ligands is essential for good performance. Use of the fibers lacking streptavidin and oligo-dT coating or fibers with covalently attached streptavidin and not the oligo-dT complexes led to poor mRNA extraction (high C_T values) and extremely poor reproducibility.

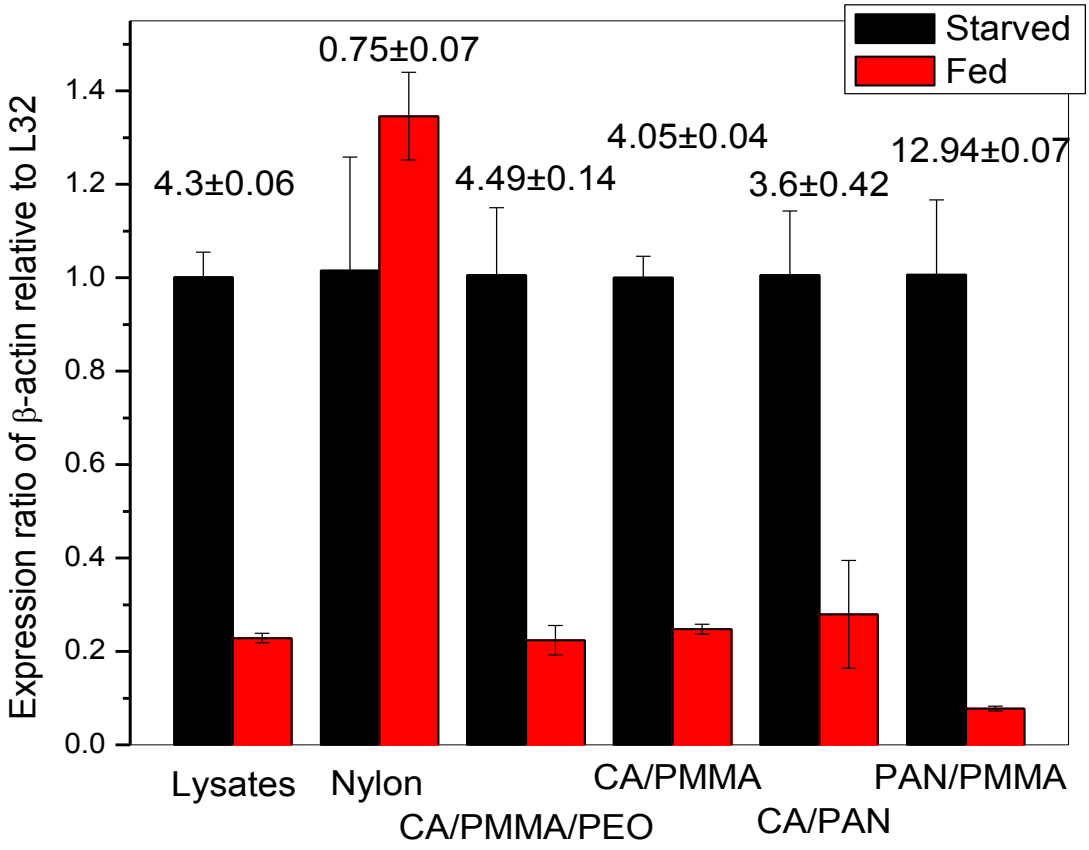


Figure 21. Performance of fiber yarns prepared from different materials. Results were obtained using mRNA eluted from 2 cm oligo dT_{25} modified yarn fragments. Numbers above the bars represent N-fold difference (mean±SD) of β-actin expression in starved vs. fed VSMC cultures (n=3).

In all experiments, L32 was used as the housekeeping gene. Comparison of β-actin expression ratios for different yarn materials to that obtained by conventional PCR of the cell lysates showed that closest correlation with conventional PCR was in the case of CA/PMMA/PEO fiber yarns. Thus fiber yarns manufactured from this material were chosen for further experiments.

Notably, conventional nylon fiber with relatively low specific surface area showed worst performance compared to other materials indicating importance of highly porous microscale architecture of the yarn. Hydrophobicity of nylon could also affect the performance of the fibers due to uncontrollable adsorption and/or partial leaching of streptavidin and streptavidin-mRNA complexes.

5.3.2. Optimization of CA/PMMA/PEO fibers

The protocol that allows one to extract mRNA from cell lysates using oligo dT_{25} coated fiber yarns was established. It was found that vigorous washing steps in the appropriate buffer[141] (1 ml of TE buffer with 0.3 M NaCl and 0.1% SDS, 2×30 min) were necessary for removing any residual physically adsorbed mRNA molecules from the fibers surface, while leaving poly-A – poly-T bonds intact. After optimization of the washing protocol, we first performed mRNA extraction from lysates obtained from a large number of cells (~10^5).

Oligo dT_{25} modified fiber yarns were incubated with these lysates to allow attachment of mRNA's poly-A tails to oligo dT. Extracted mRNA was then eluted using

DNAse, RNAse free water and reverse transcription was performed as described in the experimental section. Obtained cDNA was used in PCR amplification process and the data obtained was analyzed using double delta CT method. Relative expression of β-actin and L32 in VSMC and myocytes obtained from this experiment is shown in Fig. S3. It was found that relative expression of analyzed genes was in a good agreement with the data obtained from cell lysates using conventional PCR. There was no significant difference ($p=0.98$ for VSMC and $p=0.99$ for myocytes) between the β-actin expression ratios determined using conventional PCR and fiber-based mRNA extraction devices (Figure 22).

However, the average C_T values increased from 14±3 for cell lysates to 21±4 for mRNA eluted from the fibers. This effect was observed because not all mRNA molecules from lysates were extracted with fibers, while all mRNA molecules from total RNA are utilized in conventional PCR. Therefore, additional experiments were performed to determine sensitivity of fiber-based mRNA extraction devices.

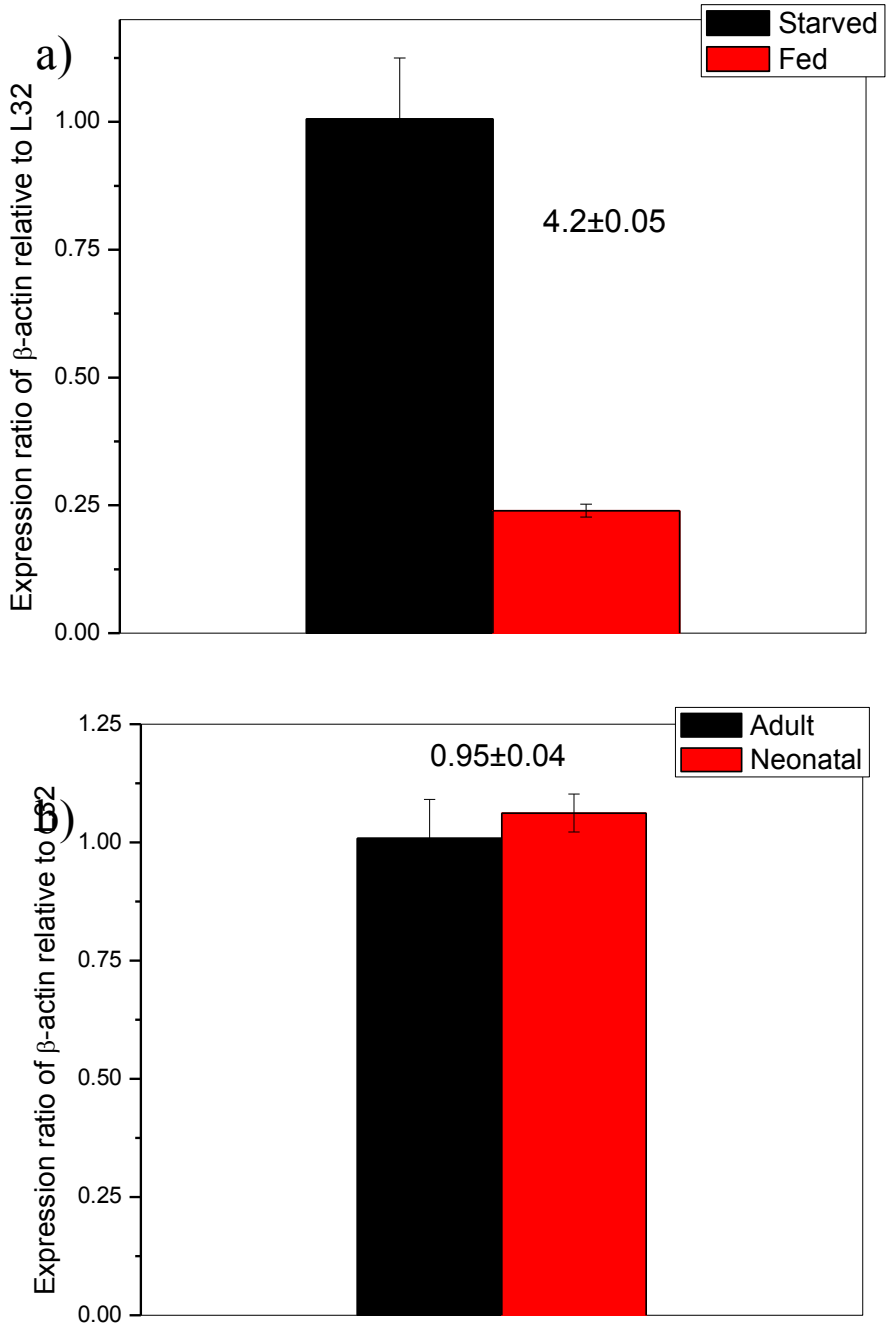


Figure 22**.** Performance of CA/PMMA/PEO fibers in two different cell culture models: (a) starved vs. fed rat VSMC and (b) adults vs. neonatal rat myocytes. Results were obtained using mRNA eluted from 2 cm oligo dT_{25} coated CA/PMMA/PEO yarn

fragments. Numbers above the bars represent N-fold difference (mean±SD) of β-actin expression in the corresponding cell culture model (n=3).

5.3.3. Extraction of mRNA from rat heart and aorta tissues

Sampling of tissues is a necessary step for determining the pathological conditions such as cancer or inflammation and is widely used in medicine. However this method could be invasive when an entire lump or whole suspicious area is removed, and therefore there is a need in developing of novel less invasive methods of analysis. Fiber-based devices could serve as an attractive candidate for this role as they could be readily modified for extraction of genetic material from the tissue of interest by just pressing them against of tissue sample. We have performed a pilot experiment for extraction of mRNA material from the freshly prepared rat tissue samples: aorta and heart samples. Fiber yarn bundles were soaked with binding buffer that is favorable for mRNA attachment to the surface of fibers. Surfactant in the buffer will help to destroy cell membranes to expose mRNA molecules from the incision surrounding tissue to the fiber yarn probe. After mRNA extraction and washing, elution and RT reaction, mRNA was analyzed by PCR analysis and following data was obtained (Table 2).

Table 2. C_T values for analysis of the mRNA extracted from freshly prepared tissue samples. (Single samples analysis. Number of replicates in PCR: N=3)

	Aorta	**Heart (incision)**	**Heart (minced)**
β-actin	23.9	24.0	22.0
L-32	21.8	22.1	19.9
Δ	2.1	1.9	2.1

It was found that the fiber-based devices are capable of extracting genetic material from crude tissue samples without any preparation steps. Data presented in the table suggests that we were able to extract more mRNA from the minced tissue, than from the plain contact of the fiber-based device with the analyzed tissue sample. This happens because more cells were exposed to the lysing buffer and provided mRNA for fiber probe to analyze. Average CT values for probing samples are almost the same, predicting good consistency of mRNA extraction using the probing method. Therefore modified fiber yarns were shown to perform extraction from crude tissue samples with relatively high mRNA extraction capacity.

5.3.4. Sensitivity experiment

To show that fiber-based mRNA extraction devices could be used for analysis of individual cells, we performed serial dilution of VSMC cell lysates. Oligo dT_{25} modified fibers were used to extract mRNA from aliquots containing genetic material corresponding to 10^7, 10^5, 10^3, 10 and 1 cell. We found that the average C_T values for mRNA eluted from the fibers after incubation with these diluted samples have increased from 21±4 (10^7 cells) to 24±3 (10^5 cells), 27±3 (10^3 cells), 30±6 (10 cells), and to 33±4 (1 cell). However the ratio between β-actin expression level by contractile and synthetic VSMCs remained almost the same as for the original cell lysates analyzed by conventional PCR (Figure 23).

The ratio between gene expressions in cell lysates was not different from the ratios obtained using the fiber-based device for mRNA extraction. A standard deviation for β-actin expression ratio increased with the dilution and was the highest for dilution corresponding to a single cell. Taken together, these findings indicate that the fiber-based mRNA extraction devices have the potential for analysis of gene expression from individual cells.

5.3.5. Comparison of fiber-based devices with magnetic beads

Comparison of performance of the fiber-based devices to the commercially available magnetic beads system was performed with low amount of total RNA, corresponding to that from approximately 100 cells. Magnetic beads utilize mRNA

extraction principle similar to that used in the fibers (binding of mRNA polyA tails to oligo-dT fragments immobilized on the bead surface), therefore such comparison was warranted. Two separate experiments for mRNA extraction were performed and average C_T values (for β-actin and L32 genes; for both fed and starved cell cultures) as well as the gene expression ratios obtained from three different methods were compared.

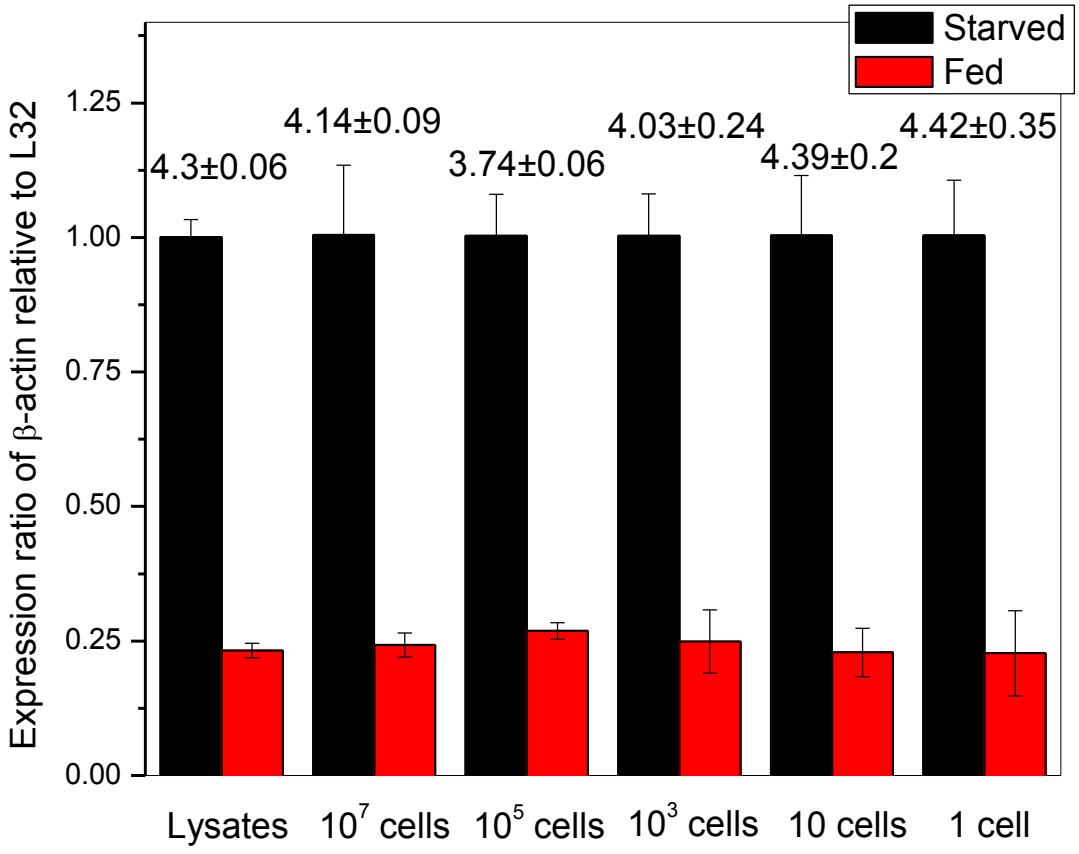
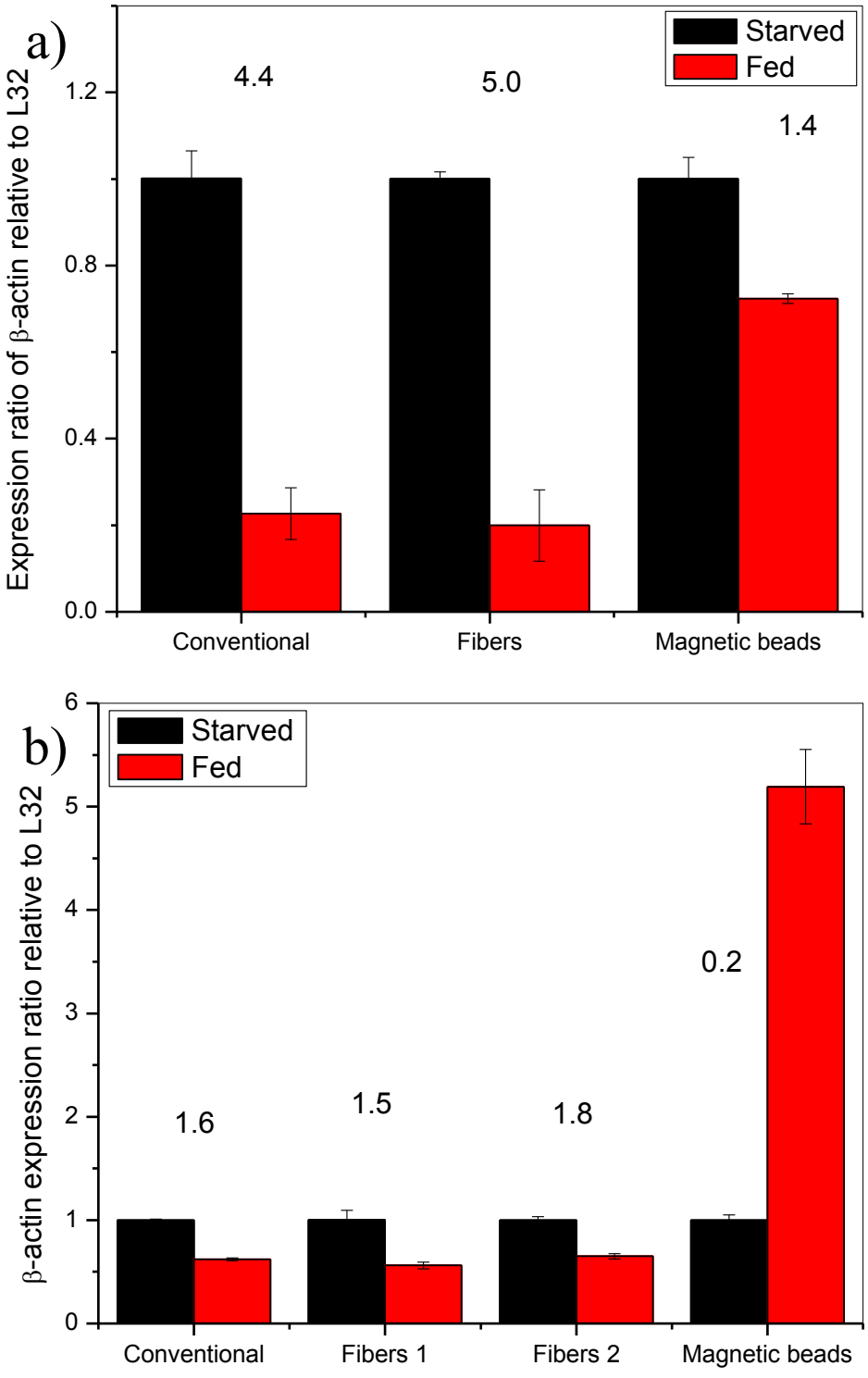


Figure 23. Performance of CA/PMMA/PEO fibers in PCR analysis of serially diluted VSMC lysates. Amount of mRNA in the final dilution corresponds to that found in one

cell. Numbers in the graphs represent N-fold difference (mean±SD) of β-actin expression in starved vs. fed VSMC cultures (n=3).

We performed analysis using fiber-based devices, magnetic beads and conventional method; the two latter methods utilized columns for total RNA extraction. Each experiment has been performed on a new batch of cell culture – separately grown fed and starved cells. Due to the difference in cell culture, gene expression ratio for the starved and fed cells could vary and we did observe change of β-actin expression ratio from 4.4 to 1.6 and 1.3 from our experiments (Figure 24 a, b, c). Relative expression ratios obtained from different methods were plotted on the graphs and compared to each other. It was found that both fiber-based devices sample replicates were capable of extracting genes in the ratio close to the standard conventional method for all experiments. At the same time, extraction of mRNA using magnetic beads was significantly different from the standard conventional method and from the fiber-based devices across all three experiments. Therefore, our fiber-based devices performed better than magnetic beads in quantitative analysis of gene extraction.


a)
Starved
Fed
4.4
5.0
1.4
Expression ratio of β-actin relative to L32
1.2
0.8
0.4
0.0
Conventional
Fibers
Magnetic beads
b)
Starved
Fed
1.6
1.5
1.8
0.2
β-actin expression ratio relative to L32
6
5
4
3
2
1
0
Conventional
Fibers 1
Fibers 2
Magnetic beads

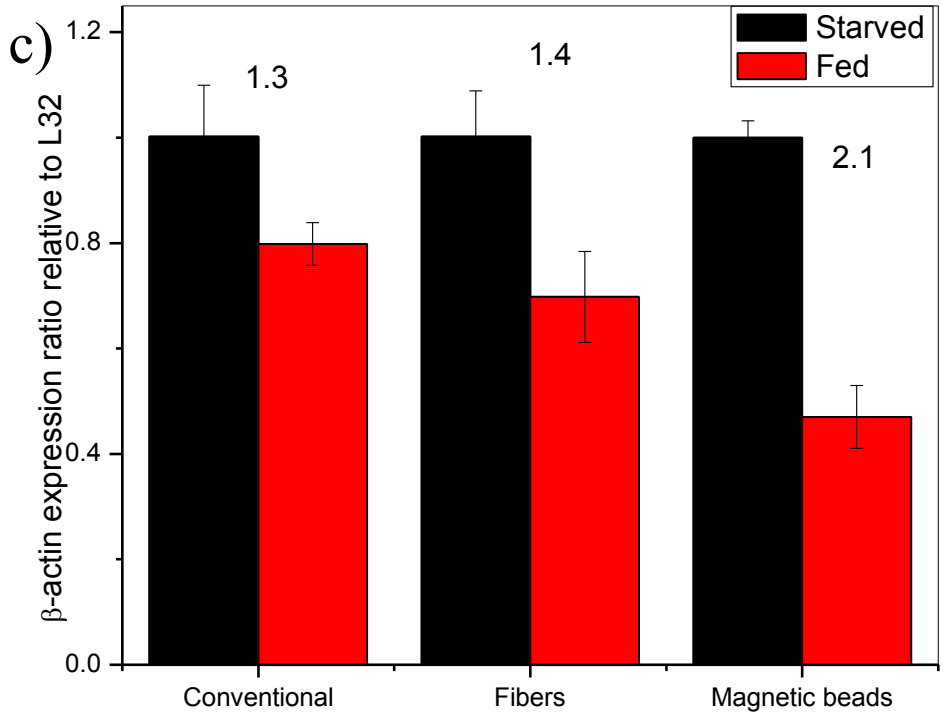


Figure 24. Relative gene expression ratios for experiments with three different cell cultures (A, B, and C) to compare performance of magnetic beads and fiber-based devices using conventional method as the "gold standard".

Another important characteristic of the mRNA extraction method is determination of the total amount of mRNA that could be extracted from samples by the method used. The conventional method extracts the whole mRNA along with total RNA and this method will indicate the maximum mRNA amount in the samples. Comparison of mRNA amount extracted from the cells was performed by comparing of averaged of C_T values across both genes and both cell cultures obtained in PCR analysis. Figure 25 provides

average C_T values with mean±SD plotted on the same graph and p values for Student T-test for paired comparison of the presented data. Student T-test was performed with the null hypothesis that sets of samples are being the equal to each other and p-values for each pair were calculated and presented in the Table 3. As it could be seen form the table, conventional method was found to extract significantly more mRNA than the developed fiber-based devices as well as magnetic beads. At the same time there was no difference in the mRNA extraction efficiency between fiber-based devices and magnetic beads in most of the samples. This data indicates that both fiber-based devices and magnetic beads are capable to extract the same amount of mRNA from the solutions.

Table 3. P-values for paired comparison of methods for mRNA extraction. N=9.

	Gene	Conventional to fiber-based devices	Conventional to magnetic beads	Fiber-based devices to magnetic beads
Synthetic (Fed)	β-actin	0.000183	0.022515	0.007876
	L32	0.001376	0.011905	0.050419*
Contractile (Starved)	β-actin	0.0448	0.032107	0.469968*
	L32	0.048448	0.006477	0.178167*

Thus, developed fiber yarn biosensors are sensitive enough to compete with the conventional methods of mRNA extraction and perform extraction of genes in correct ratio, close to conventional method. These findings suggest that fiber-based devices could be utilized for numerous applications providing excellent sensitivity and good correlation with the gold standard method.

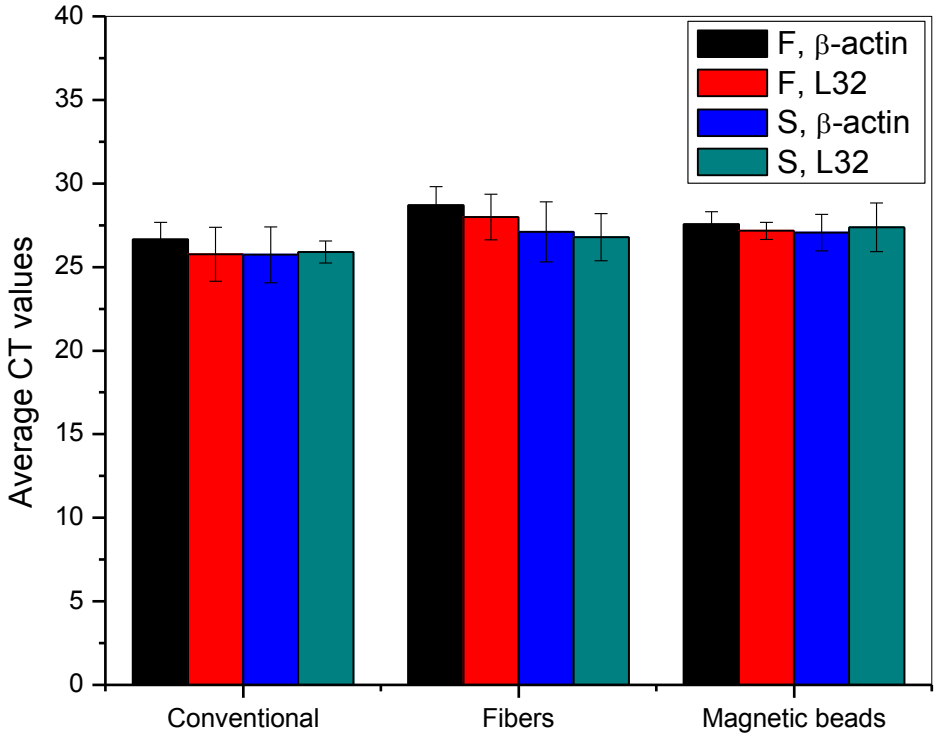


Figure 25. Comparison of mRNA extraction from starved and fed cell cultures using three methods: conventional, fiber-based devices and magnetic beads. N=9.

5.4. CONCLUSIONS

We demonstrated successful fabrication and characterization of fiber-based devices that are able to detect mRNA at the concentrations corresponding to a single cell level. Cellulose acetate fibers with PMMA and PEO outperformed other types of materials, showing the most consistent and reproducible results. This is a first successful step toward the development of highly efficient fiber-based mRNA sampling devices for gene expression analysis, confirming that the fiber yarn is able to absorb minute amounts of mRNA and following PCR provides reliable and reproducible data. Use of the fiber yarn as both sample collection probe and purification column improves the ease of use compared to conventional PCR. Further on, fiber-based mRNA sampling devices allow convenient and precise collection of genetic material from a desired area, which is determined by the diameter of the yarn and can be as small as few square microns. Overall, these fibers provide a new flexible platform for PCR analysis of extremely small amounts of genetic material corresponding to little droplets, small areas of tissue or individual cells.

CHAPTER 6

FIBER-BASED DEVICES FOR COLORIMETRIC ANALYSIS

6.1. Introduction

Enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used techniques in research and clinical laboratories of all kinds. The value of immunoassay is difficult to overestimate, as it can be used to detect a variety of molecules and proteins in the body including serum proteins, hormones, drugs, antibodies, as well as other antigens. [84-89] This method has been successfully utilized in a number of medical screening tests, and for detection of pathological conditions. [90, 91] As is the case with any assay, this method of analysis has undergone numerous enhancements since it was first described in the 1960s.

ELISA uses the specific ligands present in the body as a basis for a colorimetric assay to determine whether or not a particular antigen is present. This technique works by using antibodies which bind to the specific antigen and then introducing an enzyme capable of producing a colorimetric signal when the antigen of interest is present.

As ELISAs have evolved, several different variations of the technique have emerged. [29] Direct ELISA is based on the detection of antigen attached to the surface of well plates with the primary antibody conjugated with enzyme. Indirect method introduces secondary enzyme conjugated antibodies for the detection of primary antibodies that will react with immobilized antigens. In a sandwich ELISA assay, capture

antibodies are immobilized on the surface for detection of specific antigens or specific antibodies in solutions. [92] After antigen immobilization, solution of another primary antibody specific to the same antigen and conjugated with enzyme molecules is added – as in the direct method. ELISA is based on the detection of colorimetric product development from substrates that undergo transformation in the presence of peroxidase molecules. Color development reaction could be monitored over time in the kinetic study or just taking an end-point reading at the specific time point. This method can provide qualitative as well as quantitative data for analysis of presence of specific antigen molecules. Herein, we will refer to the methods of performing ELISA in a well plate as 'conventional ELISA'.

Conventional ELISA that is performed in standard well plates has many advantages and certain disadvantages. Numerous improvements have been successfully developed to increase sensitivity of this method. [94] The main advantage of this method is that it provides excellent sensitivity and is used for detection of analyte in solutions of extremely low concentration. On average this technique is capable of accurate measurements down to 1-10 fmol per well [95] and is sensitive to detect subtle differences in proteins and molecules. For example, ELISA is capable of distinguishing IgG antibodies with different affinities. This level of specificity with such high sensitivity makes ELISA an extremely valuable and versatile evaluation technique.

Disadvantages of this method include expensive equipment and reagents and a long time to perform the analysis. The most important of these is the extensive time

commitment required to perform an ELISA. Moreover, low sensitivity limits the efficacy of some ELISAs and an increase in sensitivity is desirable. An increase in sensitivity could limit this false positive and provide more accurate results.

Electrospun fiber yarns have shown to be an improvement by incorporating them into existing devices for bio-sensing applications. These yarns have shown positive feedback in many applications, such as possibility of single cell analysis (Chapter 5), including biosensors for detection of bacterial vaginosis. [123] Providing high specific surface area for an antigens or antibodies immobilization, fiber yarn based sensors could improve sensitivity to the method, as more sensing molecules will be immobilized on the fiber yarn surface. Inexpensive method of fiber yarns preparation and modification as well as conservation of reagents could also reduce the costs associated with development and use of ELISA assays. The total assay time for fiber yarn based sensors could be decreased due to the elimination of diffusion limited steps that take most of the time in conventional methods. Incorporating of these electrospun fiber yarns into rapid point-of-care devices will help to develop fast and simple methods for detection of highly transmitted diseases such as HIV, hepatitis, influenza. Here we propose to use CA/PMMA/PEO fiber yarns as a new platform for performing the ELISA-based colorimetric analysis.

We fabricated a simple model sandwich ELISA system and incorporated into electrospun fiber yarns. The system had similar or increased detection sensitivity and considerably reduced time of analysis compared to the conventional ELISA. This

approach opens the new potential for development of fast, sensitive and reliable immunoassay systems as it could be expanded to various analytes and applications.

6.2. Materials and methods

6.2.1. Materials

Cellulose acetate (CA, M_w = 37 kDa), poly(methyl methacrylate) (PMMA, M_w = 120 kDa) and polyethylene oxide (PEO, M_w = 1,000 kDa), Goat anti Rabbit IgG, Goat anti Rabbit IgG - Horseradish Peroxidase conjugated, Rabbit anti Bovine IgG, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich Corporation. Mouse anti TNF-α primary antibody was purchased from Abcam. Dimethylacetamide (DMAc) was purchased from Alfa Aesar. Citric acid monohydrate, 30% hydrogen peroxide, phosphate buffered saline (PBS) tablets were purchased from Fisher Scientific.

6.2.2. Conventional ELISA procedure

A model for the direct sandwich ELISA procedure was developed for use in present experiments. Figure 26 represents the schematic for the studied assay composition for both conventional analysis and the analysis using fiber-based devices for ELISA analysis. The capture antibody that was used is Goat anti Rabbit antibody IgG. We have used Rabbit anti Bovine IgG antibody as a model antigen, and the specific antibody was Goat anti Rabbit IgG antibody, conjugated with horseradish peroxidase (HRP). Conventional ELISA procedure was performed in standard polystyrene 96 well

plates. First, plates were coated with capture Goat anti Rabbit IgG antibody (1 µg/ml, 100 µl/well) and incubated at -4°C overnight. After that, plates were washed with 200 µl/well of 0.05% Tween20 in 1x PBS buffer (PBST) total of 5 times to remove any unbound antibody. Then the surface of wells was blocked using 1% Bovine serum albumin (BSA) in 1x PBS for 90 minutes at room temperature, unbound BSA was washed 5 times using the procedure described above. Different dilutions of analyte (Rabbit anti Bovine IgG) were applied to the wells and incubated for 30 minutes to allow attachment of the analyte to the capture antibody. After washing, solution of detection antibody (5 µg/ml, 100 µl/well) was added to the wells and incubated for another 30 minutes. Unbound antibodies were washed with PBST (200 µl/well) 5 times as in previous steps. 200 µl of working substrate solution of 0.6 mg/ml ABTS substrate in 0.1M citric buffer (pH=4) was added to the wells and immediately placed into the Synergy HT plate reader (BIOTEK, Winooski, VT) to read the change of absorption at 415 and 670 nm which is specific to the ABTS degradation products. Kinetic reading was done for total of 90 minutes with detection of OD change at both wavelengths every 5 minutes.

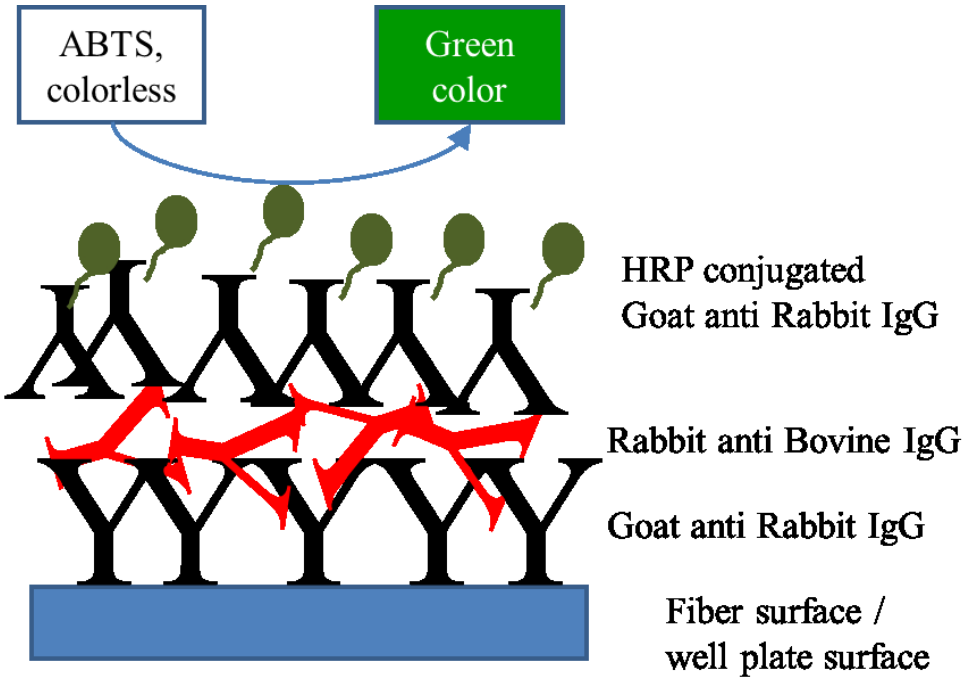


Figure 26. Schematic of ELISA analysis for fiber-based devices.

6.2.3. Fiber yarns modification procedure

CA/PMMA/PEO fiber yarns were prepared as described previously. [122] Fiber yarns were soaked in the PBST buffer solution overnight prior to the experiments to remove any contamination that could possibly be present on the surface. After overnight washing with PBST, fibers were rinsed several times with plain PBS buffer to remove any residual surfactant.

Capture Goat anti Rabbit IgG antibody was modified with sulfo-NHS-LC Diazirine, a crosslinker that will attach to the primary amines of antibody by NHS moiety. After modification of antibody with crosslinker, it was washed total 5 times from the excess of crosslinker using 30 kDa MWCO nanosep micro centrifugal devices (PALL Life Sciences). Solution of purified antibody conjugated to the crosslinker was applied on the glass slide with the fiber yarn and incubated for 15 minutes prior to UV treatment. After the incubation, UV treatment for 20 minutes was performed: diazirine group will react with C-C or C-H bonds of the fiber polymers upon activation by ultraviolet. Initial concentration of antibody immobilized on the yarn surface was equal to the concentration of the capture antibody in the conventional ELISA method. However the volume of antibody solution that was immobilized on the fibers (10 µL) was much less than that for the conventional method (100 µl). After immobilization of capture antibody, fiber yarns were washed with PBST 35 times and kept in at 4°C until future experiments.

Washing of fiber yarns was performed by pressing several times a dry folded Kimwipe paper towel against yarn pieces that were placed on the glass slide and wetted with 2 µl of washing buffer. This washing procedure allows fast and simple wash of the fiber yarns because Kimwipe will readily wick out all the liquid contained in the yarns. After that new portion of washing buffer solution (2 µl) was applied to the yarn and the wicking procedure repeated with new dry section of the Kimwipe. The number equal to 35 times of washings steps was kept consistent to allow best cleaning of the yarn pieces and maintain the same washing efficacy. Total time for the washing procedure of a single

fiber yarn piece was around 3-5 minutes and the set up allowed washing up to 10 different samples at the same time just by arranging them on the glass slide.

6.2.4 Fiber-based devices for colorimetric analysis

Fiber-based devices for ELISA-based colorimetric analysis were using fiber yarns modified as described above. The fiber yarn piece of 1 cm was incubated 10 minutes with 2 µl of the model analyte solutions – different dilutions of secondary Rabbit anti Bovine IgG antibody or control Mouse anti TNF-α antibody. After incubation, the yarn pieces were carefully washed 35 times with PBST solution using the standard method of washing. Detection Goat anti Rabbit IgG antibody conjugated with HRP solution (2 µl, 4 µg/ml) was added to the yarn pieces and incubated for another 10 minutes and then excess of the antibodies was washed with PBST. After washing, the ABTS substrate solution was added to the yarns and incubated until the coloration appeared.

6.3. Results and discussion

The colorimetric assay was based on the transformation of the colorless ABTS substrate into the green-colored oxidation products in the presence of horseradish peroxidase and hydrogen peroxide. The ABTS substrate in average possesses lower sensitivity than other HRP substrates but it has higher signal to noise ratio and develops green coloration which will be easily detectable on the white yarn surface.

6.3.1. Conventional ELISA

Standard direct sandwich ELISA was performed in a plate reader and was used to investigate the sensitivity of proposed antigen-antibodies model system. Kinetic readings of OD at 415 nm were plotted against the assay time for various concentrations of the model analyte antibody (Figure 27).

It was observed that the colorimetric signal of the substrate solution was developing rather fast using higher concentrations of Rabbit anti Bovine IgG in the samples and at the point of 5 minutes the signal was much higher than the background. Correspondingly, with the decrease of Rabbit anti Bovine IgG antibody concentration we have observed decrease of the ABTS colorimetric product development rate.

Concentration dependence of OD at 415 nm for targeted Rabbit anti Bovine IgG antibody at the 30 minutes time point is plotted in the Figure 28. Blue dash line represents the mean + 3 SD for the blank. At this time point the OD of wells for the lowest concentration of 7.5 pM becomes significantly different from the background. However this value falls into the region of average absorbance of the blank + 3 SD_{blank} and should not be used as a lowest detectable value. Therefore we have found that the sensitivity of the conventional ELISA procedure to be 15 pM concentration of the target antibody. This finding is in agreement with the standard sensitivities of ELISA method. [95] The detection limit of the developed conventional ELISA test was calculated to be in the range of 1.5 fmol per well (15 pM × 100 µl).

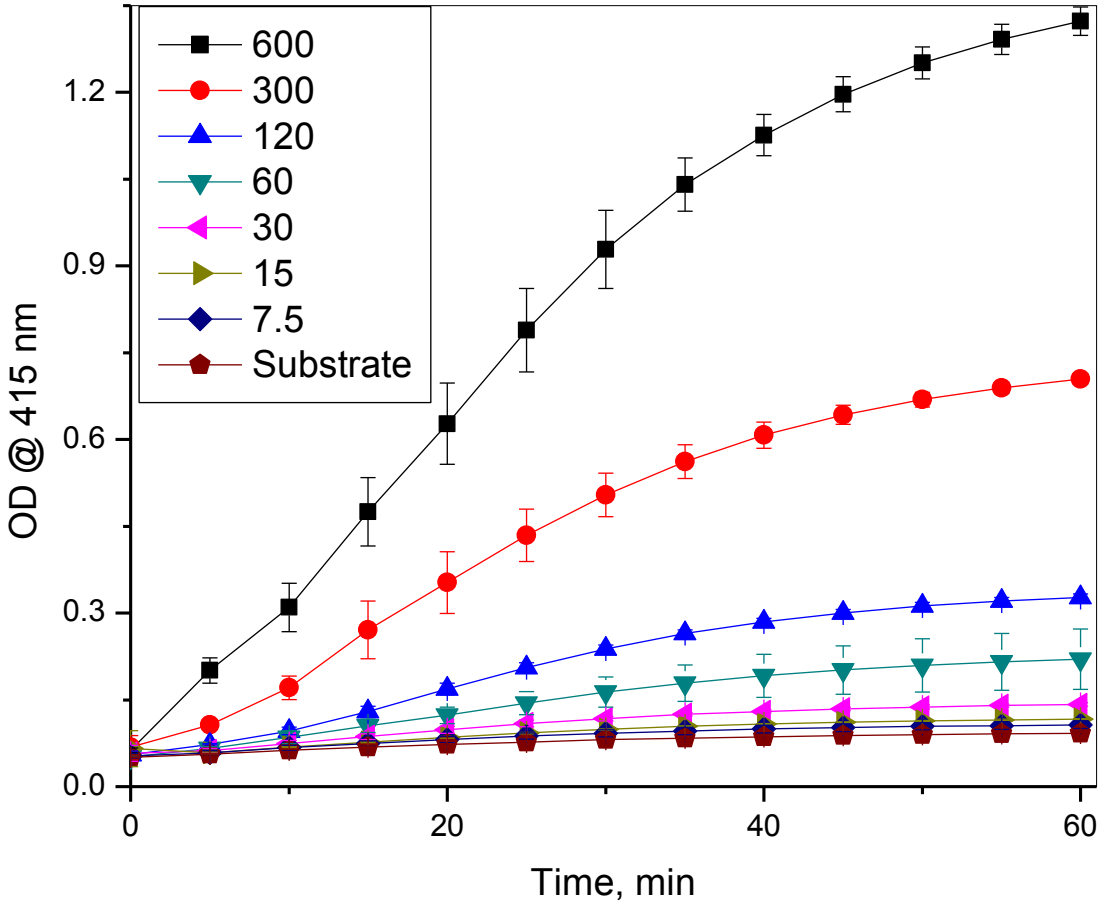


Figure 27. Kinetic curves of OD@415 nm graph for the conventional ELISA procedure. Numbers in the insert indicate concentration of Rabbit anti Bovine IgG solution in pM. (N=4)

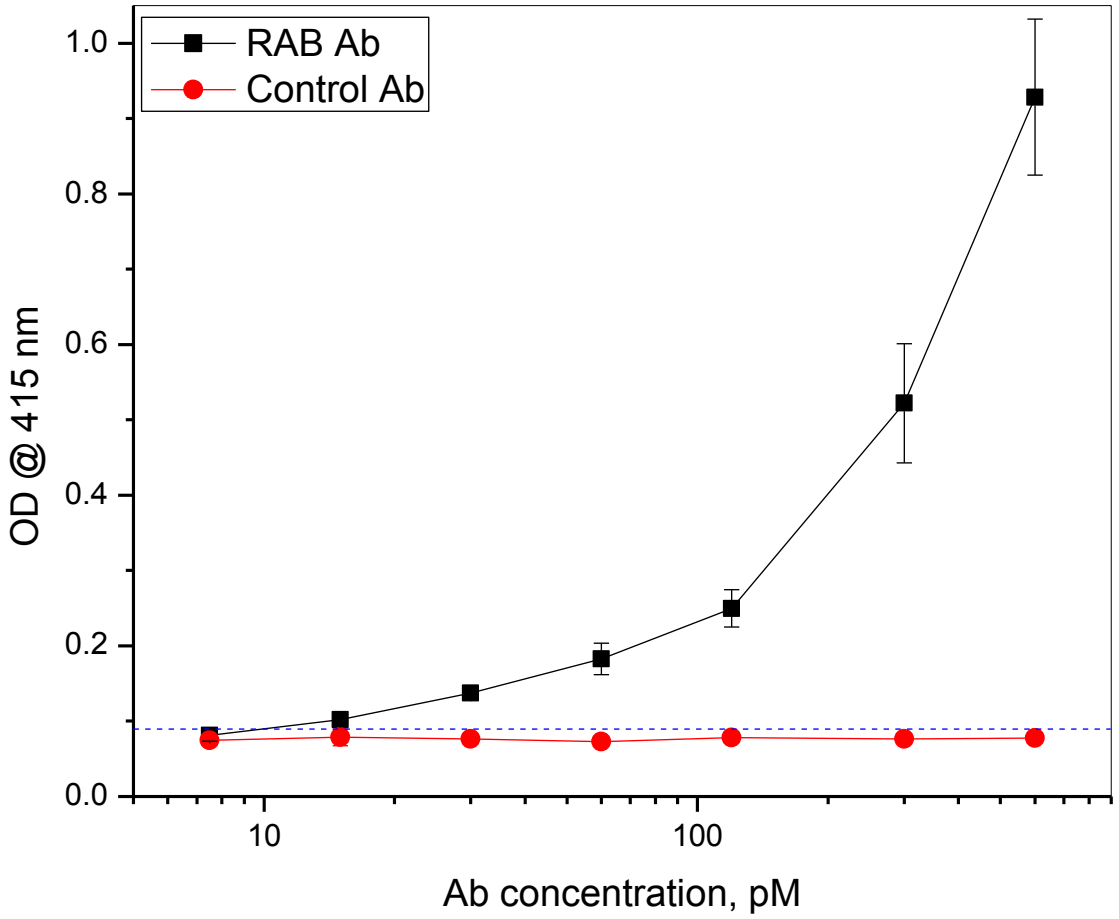


Figure 28**.** Concentration dependence of OD@415 nm at 30 minutes time point for model analyte - Rabbit anti Bovine IgG and control - Mouse anti TNF-α antibodies. The blue dashed line represents the mean + 3 SD for the blank which is a commonly used method for evaluation of sensitivity. (N=4).

6.3.2. Performance of fiber-based devices for colorimetric analysis

In the present work we have performed testing of our modified fiber yarns for the ELISA application. We performed direct sandwich ELISA procedure in the same way as for the conventional method and the scheme of overall experiment is shown in Figure 26. The experiment consisted of 5 steps: (i) modification of the yarn surface with the capture antibody (covalent attachment), (ii) blocking of the fibers surface with BSA, (iii) attachment of analyte, (iv) attachment of secondary antibody, (v) incubation with substrate for color development. Between all steps of the procedure, the yarn pieces were washed 35 times in average with 1-2 μl of PBST buffer to remove any physically adsorbed reagents. It was shown that the physical adsorption of capture antibodies on the yarn surface did not yield a detectable concentration; the antibody molecules were washed away. Therefore, the capture antibodies were attached covalently to the fiber surface. After addition of the HRP conjugated Goat anti Rabbit IgG secondary antibody to the fibers, the fiber yarns that were incubated with the test antibody will be tagged with peroxidase molecules. Upon addition of the substrate solution, the yarns will turn green in the presence of HRP.

For the highest concentrations, (from 120 pM to 2 nM), the yarns turned green very fast, in several seconds. However, for lower 60 pM concentration the coloration took about 1 minute and further slowed down with dilution of the Rabbit anti Bovine IgG content. For the lowest concentration of 7.5 pM, the detectable green coloration was achieved in 3 minutes after addition of the substrate solution to the yarn piece. Faster

coloration of yarns compared to the conventional method can be explained by an increased loading of the capture antibodies on the fiber surface.

The yarn pieces that were incubated with the targeted Rabbit anti Bovine IgG antibody and with the substrate solution are shown in Figure 29a. The fiber yarns that were exposed to the control antibodies remained not colored (Figure 29b). At least 4 replicates per test were conducted with the fiber-based devices proving the reproducibility of results. The detection limit in the fiber-based devices was found to be much lower than that provided by the conventional method. The detection limit was estimated to be around 0.015 pmol per fiber yarn (7.5 pM × 2 μl). Closer to the level of detection, the yarns coloration was not uniform. Despite this limitation, the yarns are able to detect very low concentration of the secondary antibodies. Quantification of this level of detection is however challenging.

Overall comparison of the fiber-based and conventional ELISA procedures is shown in Table 4. The total time needed for analysis of the sample contained targeted analyte is approximately 1 hour 45 minutes, from the capture and antibody immobilization to the detection. This is a great improvement of the response time over the conventional ELISA which takes more than 7 hours. This conventional ELISA followed standard total time needed for the assay but was not completely optimized. The further optimization of the conventional assay could decrease total time needed for the analysis to the level of 4 hours, which is still more than twice as long as it takes for the fiber-based tests.

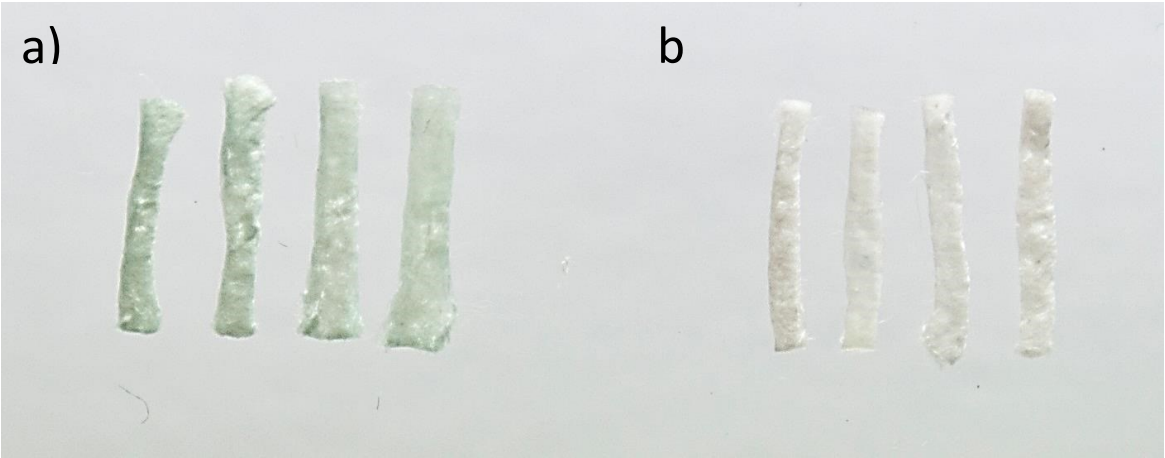


Figure 29. Fiber yarn pieces incubated with a) targeted Rabbit anti Bovine IgG antibodies, concentrations from left to the right 60, 30, 15, 7.5 pM, b) control Mouse anti TNF-α antibodies with same concentrations.

Both conventional and fiber-based devices for ELISA can be pre-manufactured and used for detection of certain common antigens (such as in the case of HIV antibodies). In this case, the time required for analysis is reduced to 40 minutes for the fiber-based devices and to 100 minutes for conventional ELISA. Moreover, fiber-based devices for ELISA-based analysis do not require additional laboratory equipment such as spectrophotometer and are much easier to perform. It is therefore possible to use the fiber-based devices for ELISA as a point-of-care method, performing analysis in the clinician's office rather than sending samples to the lab for analysis. This capability could significantly reduce costs of the analysis. Other advantage of the fiber-based probes is the

lower amount of the reagents needed for the analysis and these sensors could be used to extract the analyte from trace amounts of liquid. Finally the sensitivity of the fiber yarns was shown to be superior to the conventional ELISA method, and the total amount of analyte needed for the test is approximately 100 times lower.

Electrospun microfiber yarns provide excellent platform for the immobilization of biomolecules. High surface area provides extreme loading capacity, increasing the sensitivity limit compare to the standard methods. These fiber bundles provide a less expensive, much faster yet more sensitive way to detect various analytes in the solutions. The use of fiber-based devices for ELISA-based colorimetric analysis requires less training and could be incorporated into self-testing systems such as point of care analysis devices. Developing the method for investigation of the analyte concentrations using standards for the quantification curve we can further improve the developed method. Therefore there is an open possibility for developing of highly sensitive semi quantitative assay, the fiber-based ELISA.

Table 4**.** Comparison of fiber-based devices and conventional ELISA.

	Fiber-based devices		**Conventional ELISA**	
ELISA step	Time, min	Volume, µl	Time, min	Volume, µl
Capture antibody immobilization	30	2	240	100
Blocking the surface	30	2	120	200
Analyte attachment	15	2	30	100
Detection Ab immobilization	15	2	30	100
Detection time/ Substrate amount	3	2	30	200
Total per sample	**93**	**10**	**450**	**700**
Detection type	Naked eyes		Plate reader/ spectrophotometer	
Sensitivity	15 amol/fiber		1.5 fmol/well	

6.4. Conclusions

In conclusion, we have developed fiber-based devices for ELISA-based analysis application and qualitative colorimetric assay for antigen detection. The developed procedure allows for a several fold increase in the speed of the performed assay compared to the conventional ELISA analysis. The use of fiber-based devices requires fewer reagents to test and requires less training and no expensive equipment to perform compare to the standard method. Moreover, lowest detectable limit for this method exceeds the one for the conventional method approximately 100-fold. The lowest detection limit for concentration of model antibody was established in the range of 0.015 fmol per sample. Therefore, the proposed use of fiber yarns in ELISA-based assay systems could play a significant role in the development of novel biosensors and point-of-care devices.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Modified electrospun fiber yarns showed good performance in the application in biosensoric field for extraction of mRNA from tissues, cell lysates and up to a single cell level as well as extremely sensitive ELISA-based tests. Total of three goals were chosen for the successful development of highly sensitive fiber-based devices. First, we synthetized fiber-based devices from polymeric solutions and characterized them by several methods to investigate optimal conditions of composition and physical properties. The properties of these fibers were optimized for the application in PCR analysis, as well as for ELISA methods. We evaluated the possibility of mRNA extraction from cell lysates and tissue samples using modified fiber yarns and compared the developed method with standard techniques. Another goal was to test fiber-based devices in ELISA applications for detection of secondary antibodies from analyte solutions and comparison to the existing methods. Overall, the successful deployment of fiber-based devices in the PCR and ELISA applications leads to the development of novel highly sensitive methods of analysis and implementation in the medical diagnostics field.

Several conclusions could be made from the first goal. Different techniques for biomolecules immobilization were tested in the present work and it was found that the use of covalent attachment is more suitable for this purpose. This method of

immobilization provides stronger adherence necessary to prevent the dissociation of loaded molecules during extensive washing with surfactant solutions. Extensive washings of fiber yarns are required to prevent contamination in the downstream PCR analysis. The total specific surface area does play a significant role in the biosensors developments as it directly connects to their sensitivity. The higher surface area of fibers will increase the loading of sensing molecules on the yarns surface and therefore will enhance the sensitivity of sensors. Specific surface area of fiber yarns was calculated to be relatively high and sufficient for the immobilization of suitable amount of biomolecules. On top of that we have performed measurements of poly A molecules binding efficiency to the oligo dT_{25} modified fibers. The binding efficiency parameter is very important for the sensoric applications especially to test developed sensors with extremely low amounts of mRNA. We found that the loading capacity of the fiber-based devices far exceeded the functional requirements for mRNA extraction the chosen systems. It was found that the prepared fiber-based devices possess necessary characteristics to serve as a platform for molecules immobilization and use of fibers in biosensors development.

Initial testing of fiber-based devices prepared from different polymeric materials and modified with oligo dT_{25} complexes was performed with cell lysates. Various fiber yarn materials were tested and it was found that the CA/PMMA/PEO yarns were giving the best response in the application of mRNA extraction from cell lysates solutions. Compare to the different fiber yarn materials, CA/PMMA/PEO fibers were found to provide better batch-to-batch reproducibility and consistency in the obtained results. It

was shown that prepared fiber yarn probes could be used for mRNA extraction from various types of testing materials.

Possibility of mRNA extraction from cell lysates was shown on two different cell model systems – vascular smooth muscle cells and heart myocytes. Extraction of mRNA was also successfully performed from tissue samples, such as aortic wall or heart tissues. These findings suggest that developed oligo dT_{25} coated fiber-based devices are universal and could be used as a probing system for various applications, including biopsies or testing trace amounts of liquids/cells from investigated samples. Experiment for determination of sensitivity for proposed fiber yarn sensors demonstrated the possibility of the use for the fiber-based devices in the application for extraction of genetic material from individual single cells. More importantly, the ratio of extracted genes was found to be on the same level as it was observed for the ratio obtained from lysates prepared from high amount of cells, which indicates uniform efficiency of genes extraction.

The mRNA extraction efficiency obtained from the use of fiber-based devices was compared to the amounts of mRNA extracted using commercially available mRNA extraction system – oligo dT coated magnetic beads. It was found that there was no significant difference between these two methods in amounts of mRNA extracted. However, compare to the conventional method for extraction of total RNA both of these methods extracted lower amount of genetic material. This happened due to the fact that in the conventional method, whole amount of mRNA was analyzed and in both methods that use oligo dT coated systems mRNA was extracted partially. Overall, fiber-based

devices were found to be suitable for extraction of mRNA molecules from different systems and there was a need in exploring the possibility of application of the fiber yarns in different testing system, such as ELISA analysis.

Performance of fiber-based devices was shown in ELISA applications and semi quantitative assay for antigens detection was developed. The direct sandwich ELISA format was chosen because it was more sensitive than the simple direct ELISA method due to increased loading of secondary specific antibodies that will attach to immobilized antigen. To evaluate the efficiency of proposed fiber-based devices for applicability in the ELISA tests, we have used same ELISA schemes for both fiber-based analysis and conventional methods.

Sensitivity of the developed sensors was found to be comparable to the sensitivity of conventional 96-well based testing system. The enhanced lower limit of detection for fiber-based devices for ELISA analysis could be explained by the high specific surface area values of electrospun fiber yarn samples. Due to the higher surface, fiber yarn probes can accommodate higher amounts of capture antibodies and therefore attachment of the analyte molecules will be increased. The lowest detectable limit for the developed sensors was found to be on the level of 0.015 fmol, which is approximately 100 times lower than that for the conventional method. From the other hand, 0.015 fmol corresponds to approximately ten million molecules of the detectable antibodies.

Another advantage of the fiber-based devices is that the developed procedure allows increasing the speed of the assay several fold compare to the conventional, well-

plate-based ELISA analysis. Increase of the speed for test systems will positively affect the total throughput for developed methods. Moreover, fiber-based devices will require less technical expertise required for the performance of the assay and that could lead to developing of fast and simple types of assays that could be used at home by patients.

From the other hand, fiber yarn probes require fewer reagents per sample tested when compared to the conventional method. Reagents that are used in the ELISA analysis are mostly antibodies, which production is expensive and conservation of these reagents will definitely decrease the final costs for assays. To top the list of the cost decreasing measurements in fiber-based devices it could be mentioned that the detection for conventional system is performed by very expensive plate reader device, while the use of fiber-based devices will only require naked eyes to detect the color change.

Summarizing conclusions made from the studies of application of modified electrospun fiber-based devices, we have found that their surface could be readily modified with biomolecules and these modified fiber yarns could be utilized in various applications, two of which were described herein.

Recommendations

The present work was focused on the development of electrospun fiber-based devices for implementation in highly sensitive testing systems. While we have shown the applicability of the modified fibers in mRNA extraction from different systems such as

cell lysates and tissue samples, there is still need to prove the efficiency of these devices in extraction of mRNA from single cells. In the development of the fiber-based devices for analysis of genes expressions there is still a need in possible modifications of the developed method. Total time of the assay could be improved and the possible automation in the process of washing of fibers could be implemented. Ideally, a stand-alone device for the analysis of gene expression levels from the disposable cartridges that contain modified fiber-based devices could be designed.

Regarding ELISA applications, the procedure parameters of the fiber-based assay needs to be optimized, leading to further decrease of the total time needed for analysis. Another ELISA model systems as indirect or double indirect formats of the assay could be used for further increase of sensitivity of the developed method. Possible improvements could also be achieved in the detection of antigens by varying enzymes attached to secondary antibodies or the substrate type for development of different coloration with probably higher reaction speed. Possibility of the developing of HIV and TB testing system should be addressed because there is a need in cheap and sensitive methods for their analysis in the third world countries, where there is a lack of the sensitive equipment to use standard assay methods.

APPENDICES

Appendix A. List of abbreviations

1D – One-dimensional

ABTS – Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

BPT – Benzopyrene tetrol

CA – Cellulose acetate

CCD – Charge-coupled device

CT – Cycle to threshold

DMAc – Dimethylacetamide

DMEM – Dulbecco's Modified Eagle Medium

DNA – Desoxyribonucleic Acid

ECL – Electrochemiluminescence

EDTA – Ethylenediaminetetraacetate

ELISA – Enzyme-linked immunosorbent assay

FACS – Fluorescent activated cell sorting

FAD – Flavin adenine dinucleotide

FBS – Fetal Bovine Serum

FESEM – Field Emission scanning electron microscope

FISH – Fluorescence *in Situ* Hybridization

FLAC – *N*-fluorescein acrylamide

FRET – Förster Resonance Energy Transfer

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GOx – Glucose oxidase

HBSS – Hank's Balanced Salt Solution

HIV – Human immunodeficiency virus

HPRT – Hypoxanthine-guanine phosphoribosyltransferase

HRP – Horseradish peroxidase

IVC - *In vitro* compartmentalization

LCM – Laser capture microdissection

LSM – Laser scanning cytometry

MOCCA – Microscale Oil-Covered Cell Array

mRNA – Messenger Ribonucleic Acid

MSDS – Material safety data sheets

MWCO – Molecular weight cut off

MWNT – Multiwall carbon nanotubes

NIH – National Institute of Health

PAN – Polyacrylonitrile

PBS – Phosphate Buffered Saline

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PEG, PEO – Polyethylene glycol, Polyethylene oxide

PMMA – Poly (methyl methacrylate)

RNA – Ribonucleic Acid

RT-PCR – Reverse transcription - polymerase chain reaction

RT-qPCR – Real time quantitative polymerase chain reaction

SAMs – Self-assembled monolayers

SDS – Sodium dodecyl sulfate

SERS – Surface enhanced Raman spectroscopy

SiNWs – Silicon nanowires

SM MHC – Smooth muscle myosin heavy chain

SWNT – Single-wall nanotubes

TBP – Tributyl phosphate

UV – Ultraviolet

VSMC – Vascular smooth muscle cells

α-SM actin – α-smooth muscle actin

β-2-MG – β-2-microglobulin

Appendix B. PCR efficiency determination graph plotted for different set of primers

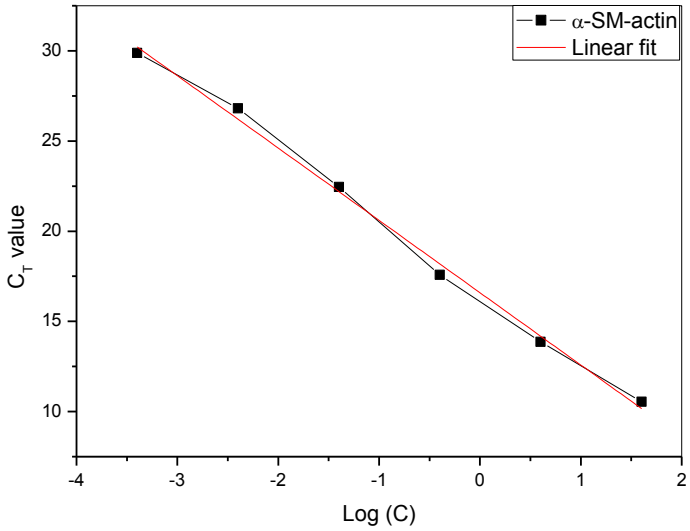


Figure A 1. CT versus log(C) graph for α-SM-actin primers.

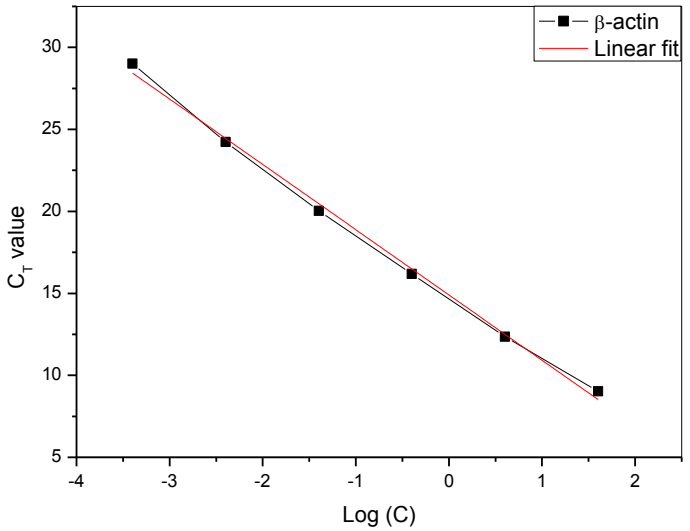


Figure A 2. CT versus log(C) graph for β-actin primers.

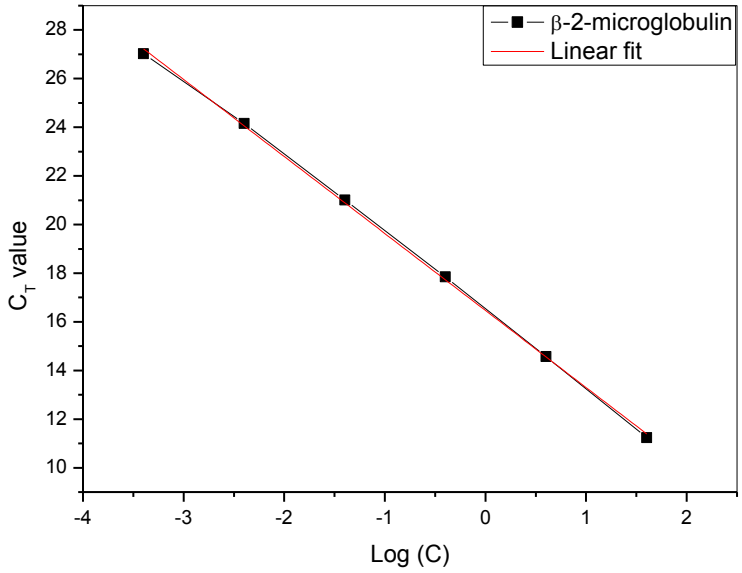


Figure A 3. CT versus log(C) graph for β-2-microglobulin primers.

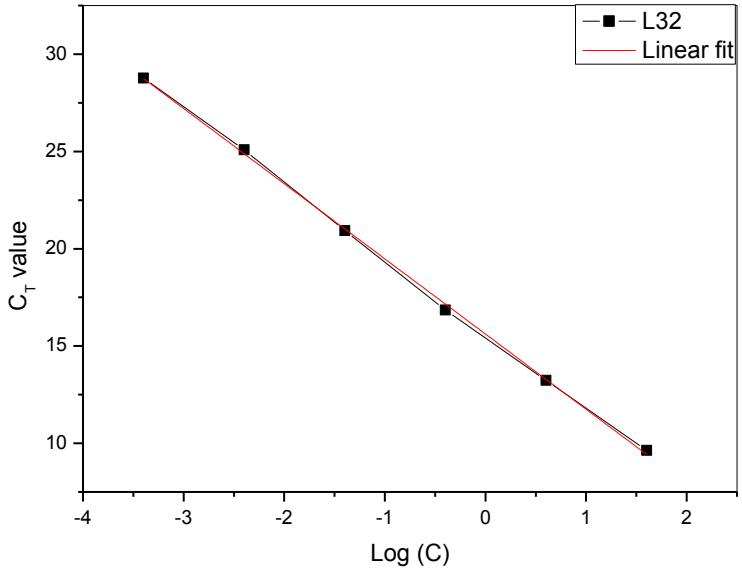


Figure A 4. CT versus log(C) graph for L32 primers.

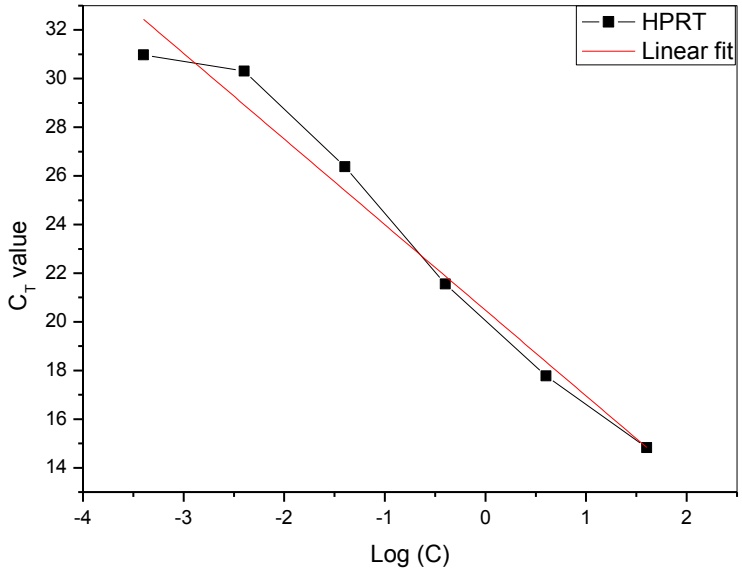


Figure A 5. CT versus log(C) graph for HPRT primers.

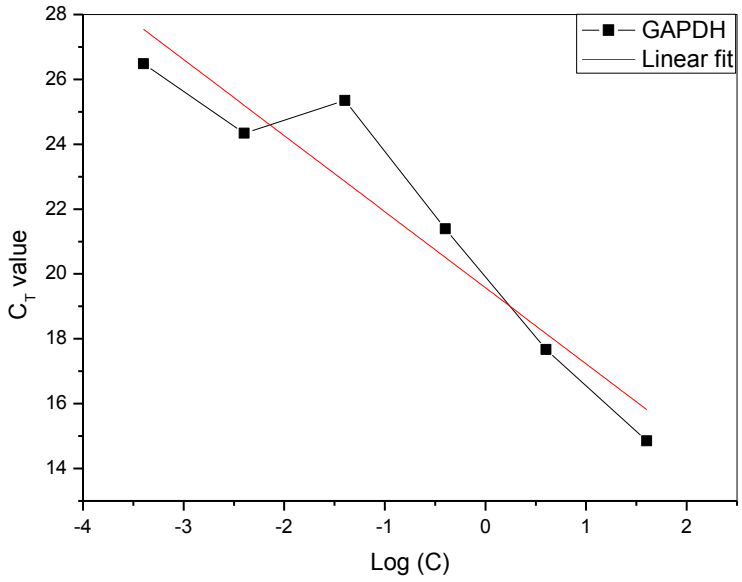


Figure A 6. CT versus log(C) graph for GAPDH primers.

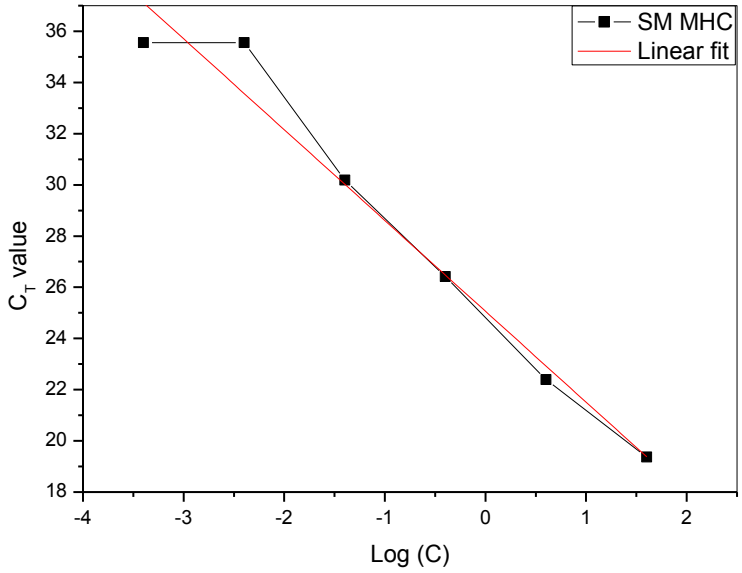


Figure A 7. CT versus log(C) graph for SM MHC primers.

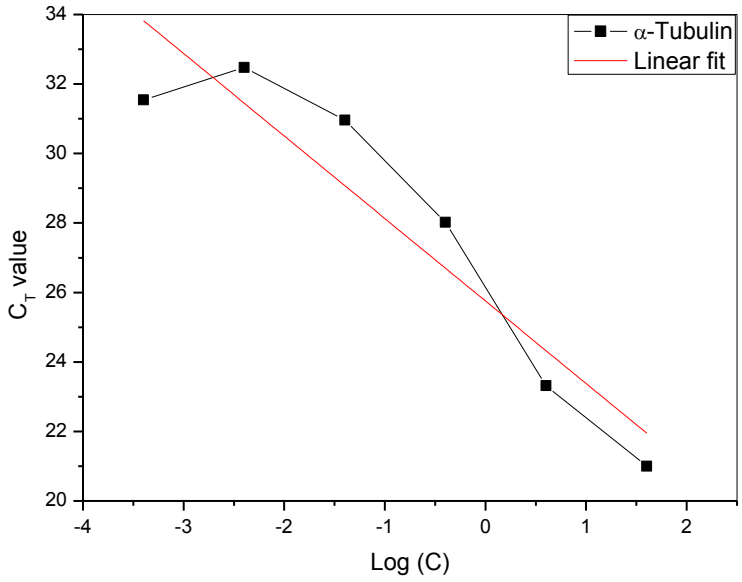


Figure A 8. CT versus log(C) graph for α-Tubulin primers.

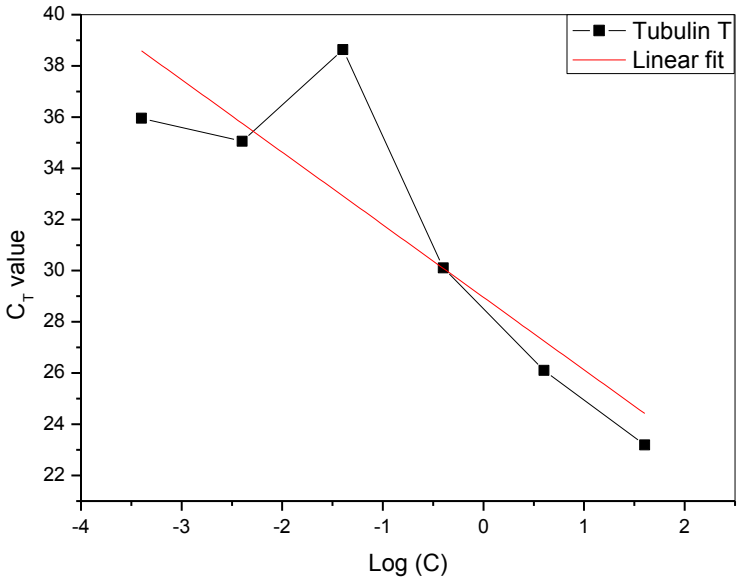


Figure A 9. CT versus log(C) graph for Tubulin T primers.

Appendix C. List of publications

Publications in peer-reviewed journals

1. Victor Maximov, Yun Xiang, Chen-Chih Tsai, Kenneth Christensen, Konstantin G. Kornev, and Alexey Vertegel "Fiber-based devices for single cell polymerase chain reaction." Biomaterials 2012, submitted.
2. Nave M, Rubin B, Maximov V, Creager S, Kornev K. Diffusion limited electrochemical formation of long nanosharp probes. Nanotechnology, 2013, Accepted.
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